Disruption of the 26S Proteasome Degradation Pathway in Mammary Epithelial Cells Results in the Induction of an Apoptotic Response

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Abstract

The mammary gland is an extremely interesting and unique tissue to study as it undergoes the vast majority of its development in the adult. It undergoes successive cycles of proliferation, differentiation and apoptosis with each pregnancy and thus is an ideal tissue in which to study questions relating to each of these processes. The aim of the work described in this thesis has focussed on isolating genes that control these processes. The technique of differential display RT-PCR was used to screen mRNA populations for significant changes in gene expression throughout pregnancy in the murine mammary gland.

As a result of this analysis, eight potential 3' cDNA fragments were identified that showed a range of expression profiles. Database searches revealed the majority of these clones to represent as yet unknown cDNAs. One clone, which was identified as the murine homologue of TBP1 was chosen for further analysis. TBP1 is a member of a family of Mg^{2+} dependent ATPases, and functions as a component of a larger multi-protein complex, the 26S proteasome. This complex acts to degrade short-lived intracellular proteins in a ubiquitin dependent manner. TBP1 expression increased around day 10 gestation in the mammary gland. Analysis of protein levels confirmed that the increase in TBP1 expression observed was due to an overall increase in the 26S proteasome. The increase in TBP1 expression observed by differential display was at the stage when the mammary gland begins to respond to lactogenic hormones by differentiating and expressing the milk protein β-casein. Studies in mammary epithelial cells (MEC) revealed the increase in the proteasome level was not lactogenic hormone dependent as the expression profile from the display results may have implied.

We further cloned murine homologues of two other ATPases, TBP7 and Sug2 and performed a yeast two hybrid assay to determine if these ATPases interacted with one another. In order to address the role of TBP1 and the 26S proteasome in the mammary gland, a series of studies using a peptide inhibitor of the proteasome were performed on MECs. These results suggested that proteasome

function was essential for MECs, and inhibition of the proteasome leads to the induction of apoptosis. This study showed that death was occurring from a specific stage in the cell cycle and that this was a p53 dependent response. To address the question of TBP1 function more specifically, a dominant negative TBP1 construct was generated. Constitutive expression studies suggested that TBP1 function was indeed essential for cell survival. The results presented demonstrate that proteasome function is an essential component of a cell's regulatory function, and that if this function is disrupted then the cell will undergo apoptosis. A greater understanding of the function of the 26S proteasome in the processes of proliferation, differentiation and apoptosis should lead to an enhanced perception of the role proteolysis plays in normal mammary gland development.

Declaration

I hereby declare that the work presented in this thesis is the product of my own efforts and is of my own composition. The work on which this thesis is based is my own except where stated in the text. No portion of this work has been submitted for another degree or qualification within this or any other institute of learning.

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Abbreviations

Amp	Ampicillin
BLG	Beta-lactoglobulin
BSA	bovine serum albumin
bp	base pair(s)
°C	degrees Celsius
cDNA	complementary DNA
cm	centrimetres
cpm	counts per minute
CsCl	Caesium chloride
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
DEAE	diethylaminoethyl
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM	Dulbelcco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
dNTP(s)	deoxynucleotide triphosphate(s)
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuidine 5'-triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetra-acetic acid
EGTA	ethyleneglyco-bis(β-aminoethylether)-N,N'-tetra-acetic acid
FITC	fluorescein isothiocyanate
FCS	fetal calf serum
g	gram
hrs	hours
IPTG	N-2-hydroethylpiperazine-N'-2-ethane sulfonic acid
kB	kilobase

kD	kilo Dalton
l	litres(s)
LB	luria broth
M	molar (moles/litre)
μCi	microcurrie
μg	microgram
mg	milligram
min	minute(s)
μl	microlitre
ml	millilitre(s)
mM	millimolar
MEM	Modified Eagle's Medium
MOPS	3-N-(morpholino) propane sulfonic acid
mRNA	messenger ribonucleic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
s	second(s)
S	Svedberg unit of sedimentation coefficient
SDS	sodium dodecyl sulphate
SDS/PAGE	sodium dodecyl sulphate/polyacrylamide gel electrophoresis
SSC	standard saline citrate
TAE	Tris/Acetate/EDTA
TBE	Tris/Boric Acid/EDTA
TEMED	N, N, N, N, tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol ($C_4H_{11}NO_3$)

UV	ultraviolet
V	volt(s)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1 Introduction

Scientific interest in the mammary gland can be dated back at least a few centuries. The first detailed anatomical descriptions of the breast and its blood supply were described in the 19th century. This settled the details of the gross anatomical structure of the gland but the later introduction of the light microscope produced a number of misconceptions about gland secretion. Examination of sections of lactating mammary gland led early histologists to suggest that secreted milk consists of the tips of the mammary cells themselves, which broke off into the lumen of the gland. This misconception was later dispelled and a firm understanding of gland development and milk secretion was established at the start of this century. Recent advances such as the introduction of the electron microscope, and the pioneering animal studies by endocrinologists in the first half of this century have enhanced our knowledge of this structure. This early work provided the basis for the description of the biochemical pathways involved in gland development and has led the way to a new generation of studies on mammary gland development and function. This work is now based on genetic manipulation of these pathways to elucidate definitive roles for the genes known to be involved in mammary development.

The mammary gland holds great interest to biologists as it is in the unique position of being a structure that develops almost exclusively in the adult. The gland is a feature unique to mammals and its prime purpose is to synthesise and secrete milk for the nourishment of mammalian young. Scientific interest in the mammary gland arises from two fronts. The mammary epithelial tree is a site of neoplastic transformation and therefore is of great interest to cancer biologists. Normal development of the gland is of interest to developmental biologists as the mammary gland is a tissue that has the ability to undergo successive rounds of proliferation, differentiation, and apoptosis. It is therefore one of the few tissues in which questions relating to all three of these processes can be addressed. Determination of the cellular pathways through which this process of organ growth

and differentiation occurs may lead to a better understanding of why the mammary gland has such a high neoplastic transformation potential.

1.2 Mammary Gland Morphology

1.2.1 Embryonic Development

The functional and structural development of the mammary gland can be divided into distinct stages. These include fetal growth, growth of the gland at puberty, development of the gland during pregnancy and lactation, and involution of the gland following weaning (Figure 1.1). Although the major part of gland development occurs during pregnancy the organ is already formed in the embryo. Embryonic development of the gland is influenced by steroid hormones and signals from the surrounding mesenchyme. In mouse embryos the anlage of the mammary gland is visible at around day 11.5. This develops into a small epithelial bud on day 12.5 which increases in size slowly until it reaches the resting period (day 15.5). At day 16 a primary sprout begins to grow into the mammary fat pad precursor tissue. Mammary rudiments form in both sexes, but by day 14 in male embryos the mammary mesenchyme condenses around the epithelium and causes detachment of the gland from the epidermis. This process is associated with massive necrosis in the epithelium and this destruction is driven by androgens (Kratochwil, 1977). Ceriani (1970) demonstrated that embryonic rat mammary glands (day 16) had the potential to respond to lactogenic hormones in culture. These glands were seen to express a casein-like material e.g. a phosphoprotein which comigrated with casein. Two tissue compartments are involved in mammary development: the parenchyma which is the epithelial component, and the stroma composed of connective tissue elements. These two compartments are separated by a basement membrane that consists of extracellular matrix materials (Imagawa *et al.*, 1990). Morphogenesis of the mammary gland is strongly influenced by the mesenchyme

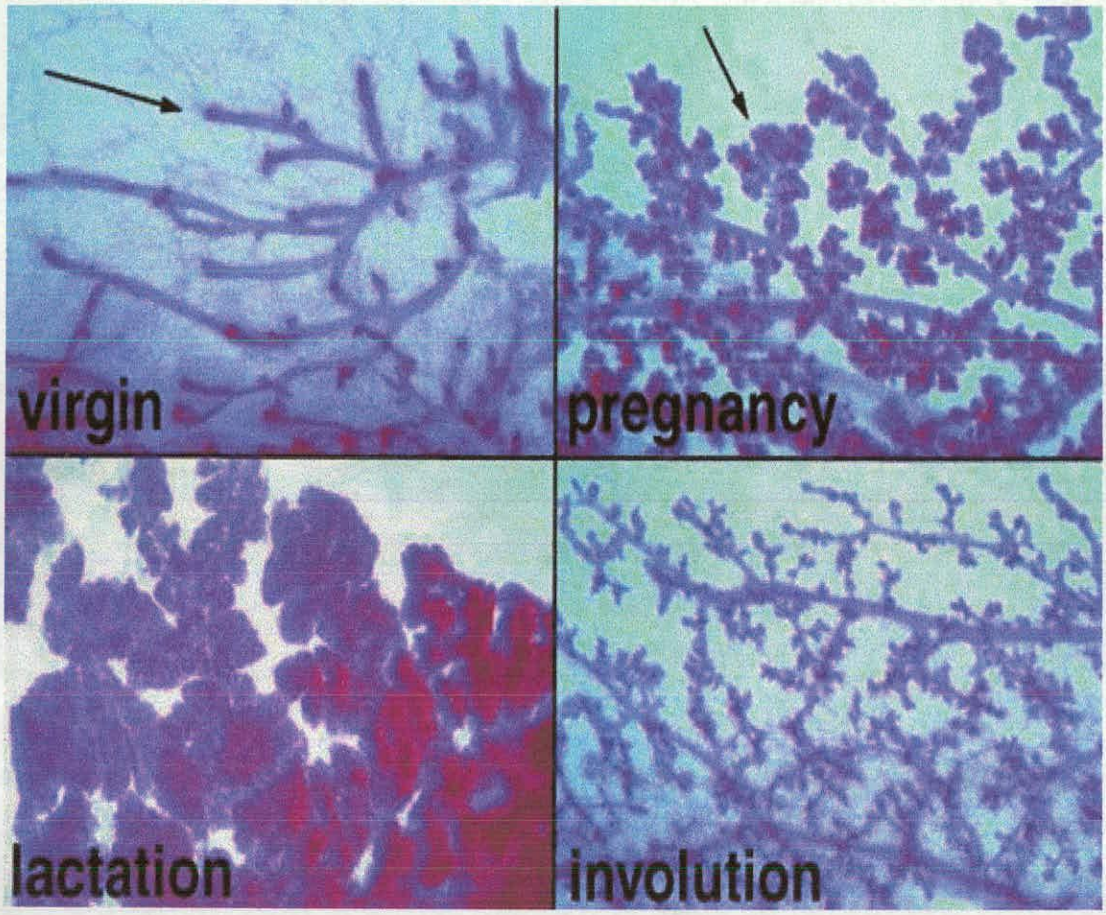

Figure 1.1 Whole Mount Sections of Murine Mammary Gland. Whole mammary gland from virgin, pregnant. lactating, or involuting mice were fixed and stained to show the developing mammary glands in these mice. This figure was obtained from the Biology of the Mammary Gland Web Page (http:\\.mammary.nih.gov).

and epithelial-mesenchymal interactions have been observed during embryonic development. The gland interacts with two different types of mesenchyme: a mammary mesenchyme surrounding the epidermal bud which contains steroid receptors, and the future fat pad which consists of preadipocytes. At birth in the mouse the mammary tree consists of up to 20 branches.

1.2.2 Postnatal Development

In postnatal life the mammary parenchyma consists of lumina surrounded by single or multiple layers of epithelial cells with a basal layer of myoepithelial cells. The luminal epithelium is specialised for the synthesis and secretion of milk proteins and is organised into structures called ducts, terminal end buds (TEB), alveoli and lobules. Major ducts serve as channels and reservoirs for the milk during lactation. Terminal end buds are specialised groups of cells at the ends of ducts that function as the sites of cell proliferation in the growing gland (Figure 1.2). Alveolar cells, present in resting adult glands and during pregnancy, are the prime components of milk synthesis during lactation. The clusters of alveoli developing during the later stages of pregnancy are called lobules. Myoepithelial cells form an almost continuous sheath around the major ducts and contract in response to oxytocin during lactation. This results in the alveoli releasing milk into the mammary ducts (Pitelka, 1978).

The extent of mammary gland growth between birth and puberty is limited to a low level of ductal elongation and branching. The growth rate of the mammary gland at this stage is comparable to the overall growth of the animal. Accelerated ductal growth commences with the start of puberty and it is at this stage that terminal end buds are first observed. The terminal end buds are specialised structures at the end of the growing ducts. They consist of two distinct cell types, the body cells that give rise to mammary epithelial cells, and the cap cells which are the precursor of myoepithelial cells. This pattern of growth continues until the constraints of the fat pad are met, and at this stage the proliferative rate is

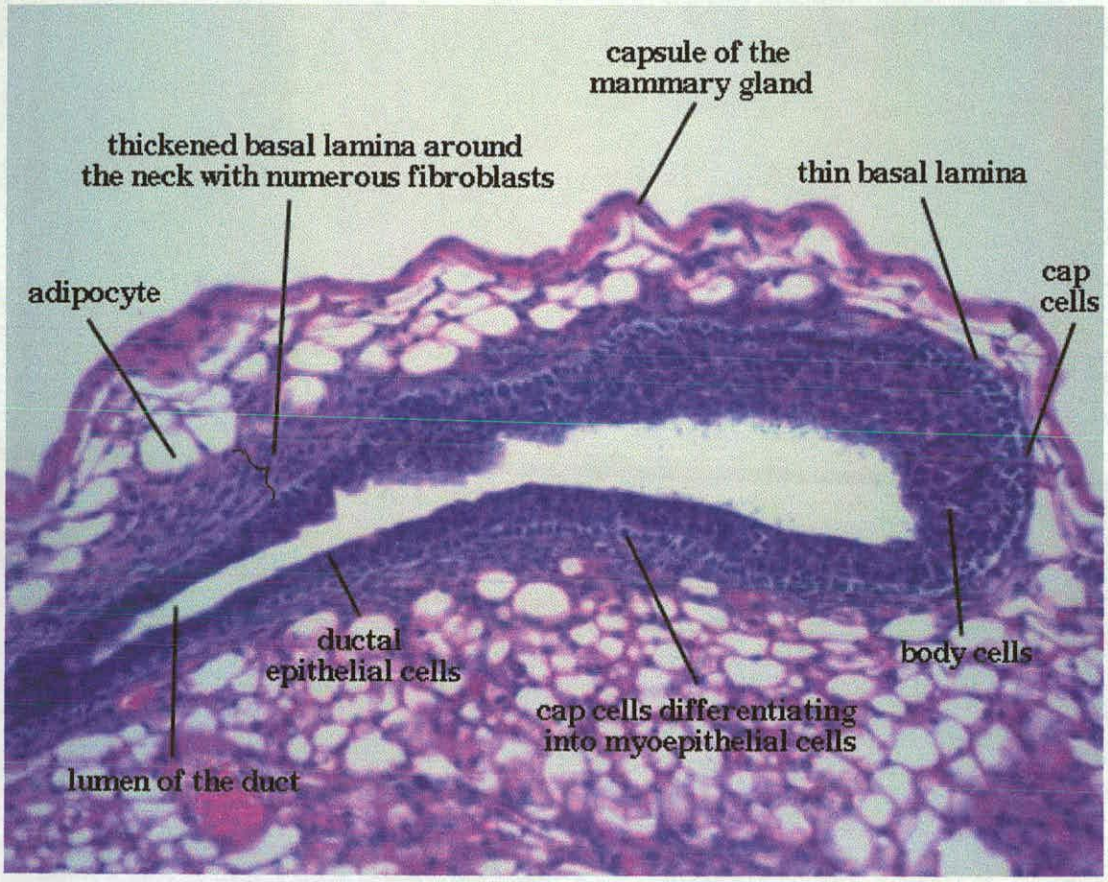

Figure 1.2 Mammary Gland Terminal End Bud. This is represented by a section through the terminal end bud of the gland. The relative positions of the cap and body cells are shown. The cap cells differentiate to give rise to the myoepithelial cells. The terminal end bud is surrounded by a basal lamina composed of fibroblasts and an outer layer of adipocytes. This figure was taken from the Biology of the Mammary Gland Web page (http:\\mammary.nih.gov).

significantly reduced (Williams and Daniel, 1983). In mice mammary gland growth plateaus at around 5 to 6 months of age. At this stage DNA synthesis is observed only within the alveolar cells suggesting that this is the renewing cell population in the adult. Following puberty mammary gland growth results in a highly branched ductal system. Variations in mammary epithelial cell proliferation have been observed during the oestrous cycle. In general the most rapid DNA synthesis occurs during diestrous when the glands are the least developed (Bresciani, 1971).

1.2.3 Development During Pregnancy

The most dramatic changes observed in the mammary gland occur following the onset of pregnancy. Profound cell proliferation occurs where the interductal spaces seen in the virgin are gradually filled due to increased branching and lobuloalveolar development. Cell division during pregnancy occurs within both the alveolar and ductal cell populations. Maximal rates of division are observed early in pregnancy, around day 5 of gestation, the stage of implantation of the embryo (Bresciani, 1971). Proliferation within these cellular compartments continues throughout pregnancy and also during early lactation. Terminal differentiation of alveolar epithelial cells is completed at the end of gestation and this process results in the onset of milk secretion at parturition. Distinct steps of cellular differentiation take place during pregnancy and lactation and are defined by the sequential activation of milk protein genes. Low levels of milk proteins can be detected in virgin mice during oestrous and their activation increases dramatically during pregnancy. WDNM1 mRNA increases in early pregnancy (day 5), which is following by an increase in β-casein mRNA around mid pregnancy (day 10). WAP mRNA is known to be expressed in the later stages of pregnancy (day 15) and α-lactalbumin mRNA is expressed just prior to parturition (Robinson *et al.*, 1995). The activation of these milk protein genes can be used as markers of the early proliferation of the gland (WDNM1), the commencement of differentiation (β-casein), terminal differentation (WAP), and the attainment of a secretory ability (α-

lactalbumin).

The ability to produce milk is the primary characteristic which divides mammals from any other class of species and allows birth to occur at a relatively early stage of development. At parturition a series of changes occurs which transform the alveoli into a fully secretory state. The majority of the studies in lactogenesis have been performed on ruminants and these have divided this process into two stages. The initial stage occurs during pregnancy when the gland is sufficiently differentiated to secrete small quantities of milk components, such as casein and lactose. This early secretion product is called colostrum. The second stage of lactogenesis is defined by the onset of copious milk secretion at parturition. It is triggered by a decrease in circulating levels of progesterone and a maintainance of prolactin concentrations (Linzell and Peaker, 1971). Removal of the milk from the alveoli is achieved by contraction of the myoepithelial cells surrounding the alveoli and ducts. The composition of milk varies dependent on the species examined. In rodents the major milk proteins are the caseins and whey proteins. α- and β-casein are involved in micelle formation which act to transport minerals within milk. The whey proteins are α-lactalbumin and whey acidic protein (WAP). The exact function of WAP is unclear at present but the correct temporal expression is a prerequisite for differentiation of the mammary gland (Burdon *et al.*, 1991). α-lactalbumin is a calcium metalloprotein which is involved in lactose synthesis.

1.2.4 Gland Remodeling

Following lactation the mammary gland undergoes a remodeling stage called involution which results in a restructured gland. Involution is characterised by a co-ordinated process of alveolar programmed cell death (apoptosis) and lobular-alveolar remodelling. This is an extremely rapid process and in rodents the mammary gland can be restructured completely within a few days following the initiation of apoptosis. The triggers for involution have not yet been identified but

potential candidates are the removal of lactogenic hormones induced in response to suckling, and an accumulation of apoptosis-inducing factors in the unsuckled milk such as TGFβ related molecules (Streuli *et al.,* 1997). Involution of the mammary gland occurs in two distinct phases. The initial phase involves the apoptosis of alveolar cells. This apoptotic process start at around day 1 after weaning, peaking on day 3 and is followed by a subsequent decrease in the rate of apoptosis. The apoptotic process is characterised by a number of morphological changes. These commence with a loss of cell junctions and other specialised plasma membrane structures such as microvilli. At the same time the nuclear chromatin condenses and divides into one or several large masses at the nuclear periphery. The nucleolus disaggregates and the nuclear membrane adopts internal folds. The nucleus may subsequently split into several fragments. Membrane blebbing then follows and the cell ulimately breaks down into apoptotic bodies which are phagocytosed by neighbouring cells (Wyllie *et al.,* 1980). The apoptotic process is a relatively short reaction, which *in vitro* was shown to occur in approximately one hour. Apoptosis is not restricted to the involution period of gland development. Humphreys and co-workers (1996) have observed a significant rate of apoptosis in the body cells of the terminal end bud. A spacial restriction of apoptosis was shown to occur suggesting that apoptosis may function to maintain the structure of the terminal end bud and also play a role in the formation of the duct in the developing mammary gland. During the second stage of involution the lobular-alveolar structure of the gland is destroyed as matrix degrading metalloproteinases degrade both the basement membrane and stromal ECMs. Both of these stages of involution are characterised by changes in gene expression or activity. The net result of this process is a replacement of most of the epithelial cells by adipose tissue and a gland which is morphologically very similar to that observed in the virgin animal.

An essential feature of the mammary gland is its ability to regenrate a functional epithelium following cycles of lactation and involution. This would suggest that progenitor or stem cells exist within the gland. It is well documented that any part of the mouse mammary epithelial tree upon implantation into a cleared fat pad can reproduce a function mammary gland with the correct structures present.

Williams and Daniels (1983) demonstrated a population of undifferentiated cells present in the outer layers of the developing TEB. These cells, the cap cells are thought to be one type of stem cell present in the mammary gland. Recently Kordon and Smith (1998) have demonstrated that an entire functional mammary gland can be produced from a single cell. The experimental model involved infecting mammary epithelial cells with mouse mammary tumour virus and performing serial transplantation of clonally derived outgrowths. Second generation glands were observed to contain the same viral insertion sites providing evidence for self-renewal of the original stem cell. This study estimated that a single mammary epithelial stem cell may have the capacity to produce approximately 10^{12}-10^{13} multipotent, proliferation-competent offspring before reaching proliferative senescence. These observations suggest that mutations in this stem cell population may be responsible for early manifestations of malignancy and for this reason are of great interest to the study of mammary gland development.

1.3 Experimental Manipulation of the Mammary Gland

The following sections describe in detail some of the effects specific genes have on mammary gland development. The mammary gland undergoes the vast majority of its growth postpubertally and this enables experiments on development to be performed on juvenile or adult glands. The following is a brief description of the three major technologies using to study mammary gland development. Defined amounts of growth factors have been mixed with ethylene/vinyl acetate copolymer to produce a hard pellet, which can then be surgically implanted in small pieces (<1mg) directly in front of the growing ductal tree (Daniels *et al.*, 1972). This method when combined with morphometric analysis can be used to determine the response of specific cell or tissue types to the administered factors. Recent advances by Edwards and colleagues (1996) have utilised transplantation methodologies to determine the effects of overexpressing a particular gene of

interest on gland morphogenesis and proliferation. Using this method the gene of interest is introduced into primary cultures of mammary epithelial cells using a retroviral vector. These cells are then transplanted into a cleared mammary fat pad where they reform the epithelium. Some of these cells will subsequently express the introduced gene. Insights into development can be examined by inappropriate expression of genes using this method. It is also particularly useful in examining the neoplastic changes caused by the introduction of a single oncogene. Tissue specific transgenic or knockout mice have been generated using the promoters of milk protein genes. In particualr the WAP promoter and the promoter of the ovine milk protein gene, β-lactoglobulin (BLG) have been used extensively. This approach directs overexpression of the gene of interest specifically to the mammary epithelium where the effects of a particular gene can be examined. This is particularly useful when the gene of interest may cause embryonic defects when deleted or overexpressed in the whole animal. In some cases when a transgenic animal is produced, if the animal is compromised due to this gene disruption then very little mammary tissue exists. This is mainly due to loss of fat from the gland and complicates examination of any mammary gland phenotype. Directing expressing to the mammary gland itself overcomes some of these problems. More recently a number of systems have been described which allow inducible expression or deletion of a gene. These include the tetracycline system (Gossen *et al.*, 1995) or the Cre-Lox system (Lakso *et al.*, 1992, Pichel *et al.*, 1993). The major advantage of these systems is that they allow the effects of disruption or deletion of a specific gene to be examined at a particular stage of development. They are particularly useful when the gene to be examined is known to be essential for embryonic development. Expression of the mutated gene later in development allows the effects of the gene to be examined in the developed animal. These technologies have been developed in part due to the uniqueness of the gland and have provided a basis for specialised approaches to study developmental, neoplastic and functional studies in the mammary gland.

1.3.1 Hormonal Regulation of Mammary Gland Development

1.3.1.1 Oestrogen

The role that systemic hormones play on mammary gland development has been recognised for some time. More recently the availability of transgenic mice has identified the precise roles that these hormones play *in vivo* in mammary gland development. Oestrogen has been described as playing a major role in ductal elongation in the gland. The observation that ductal outgrowth was greatly accelerated during pregnancy led to this suggestion but more conclusive proof was obtained when mice deficient in the oestrogen receptor (ER) α gene were produced (Korach *et al.*, 1996). Analysis of the mammary glands of females with this deletion at 4 months of age revealed the presence of a primitive ductal rudiment devoid of TEBs, whereas a fully developed ductal tree was observed in wild-type siblings. Mammary transplantation experiments revealed that ductal outgrowth was dependent on the presence of ER in the stroma but not in the epithelium. The data from these mice suggested that although ER are detected in both the epithelial and stromal compartments of the mammary gland, epithelial morphogenesis induced by oestrogen is a stromal mediated event and that the epithlial ER is not necessary for oestrogen induced mammary epithelial proliferation (Cunha *et al.*, 1997).

1.3.1.2 Progesterone

Progesterone functions in preventing lactogenesis by maintaining high circulating levels until just prior to parturition. Females lacking progesterone receptors (PR) show more severe reproductive defects than the ER deficient mice. Due to this the effects of progesterone on mammary development was studied on ovariectomised PR deficient animals by giving exogenous oestrogen and progesterone. The addition of both of these hormones to control animals resulted in extensive arborization of the ductal tree and some alveolar development, whereas this was virtually absent in the PR deficient mice (Lydon *et al.*, 1995). The function

of the PR is mainly linked to the epithelium in contrast to the ER, which exerts its effects through the stroma.

1.3.1.3 Growth Hormone

Slow release implants of growth hormone (GH) have also been shown to regulate TEB and ductal growth (Dembinski and Shiu, 1987). Indeed the stimulation of mammary development at puberty is a GH dependent event. This GH effect is thought to be mediated through insulin-like growth factor-1 (IGF-1), produced in the stromal compartment (Kleinberg, 1997). A synergistic action between IGF-1 and oestrogen results in TEB development. Growth hormone receptors are members of a large haematopoietin receptor superfamily. These receptors, which include the prolactin receptor, signal through a series of tyrosine kinases to stimulate transcription of target genes. This signalling pathway will be described in detail for the prolactin receptor.

1.3.1.4 Prolactin

Prolactin was first purified in 1933 and has been shown to regulate gonadal and behavioural functions, and it also exerts multiple effects on the immune system (Henninghausen *et al*, 1997). The best characterised role that prolactin plays is in the proliferation and functional differentiation of lobulo-alveolar structures in the mammary gland during pregnancy and to stimulate lactogenesis. These properties are mediated through the activation of genes involved in growth control and differentiation. In additional to the role prolactin plays in the differentiation of the mammary gland, a mitogenic role has been described. Prolactin is synthesised in the lactotrophic cells of the anterior pituitary. Its signalling pathway is described in section 1.4.1. The establishment of the prolactin pathway has come from details elucidated from mice deficient in some of these components. Deletion of the prolactin gene results in ductal arborization in adult virgins suggesting a role in ductal morphogenesis during puberty (Horseman *et al.*, 1997). As prolactin deficient mice do not ovulate the effect observed may be indirect. Females with

only one intact prolactin receptor allele fail to lactate after their first pregnancy (Ormandy *et al.,* 1997). This suggested that epithelial proliferation and differentiation during pregnancy requires a threshold level of prolactin receptor which cannot be obtained with just one functional allele. Mammary gland development in these mice was normal in any subsequent pregnancies suggesting that continued hormonal stimuli leads to a functional gland. It is currently unknown whether other growth pathways can compensate for a decreased prolactin receptor population.

1.3.1.5 Oxytocin

Oxytocin is a hormone that acts to regulate parturition and lactation. It induces the contraction of myoepithelial cells surrounding the alveoli and thus induces milk ejection. Oxytocin knock-out mice were shown to be fertile and able to deliver pups, but the pups died within 24 hours due to lack of milk. This could be reversed with an exogenous injection of oxytocin which restored milk ejection in response to suckling. These mice show a partial involution during lactation despite suckling and the presence of lactogenic hormones (Wagner *et al.,* 1997). These studies suggested a role for oxytocin in post-partum milk ejection and also alveolar cell proliferation.

1.3.2 Growth Factor Regulation of Gland Development

1.3.2.1 Transforming Growth Factor Beta

The transforming growth factor β (TGFβ) family play a role in mammary development. TGFβ is a secreted growth and differentiation factor that exists in three different isoforms. It exerts a range of biological functions that are dependent on the target cell. Interaction of TGFβ with its cell surface receptors results in

either a stimulation or inhibition of cell proliferation. Its antiproliferative effects are best documented on epithelial cells, whereas the stimulatory activity affects mainly cells of a mesenchymal origin such as fibroblasts. TGFβ has also been observed to effect cell differentiation, which is thought to be related to its ability to modulate cell interaction with the extracellular matrix. TGFβ is widely expressed during development and its biological activity has been implicated in epithelial-mesencymal interactions e.g. in branching morphogenesis of the lung, kidney and mammary gland (Silberstein and Daniel, 1987; Robinson *et al.,* 1991 and Rogers *et al.,* 1993). All three TGFβ isoforms are expressed in the mammary gland although their expression patterns overlap in both the epithelial and mesenchymal compartments. Exogenous TGFβ was observed to inhibit both the formation and growth of ductal buds and to stimulate production of extracellular matrix. Overexpression of active TGFβ1 in the mammary glands of transgenic mice resulted in the formation of a hypoplastic ductal tree (Pierce *et al.,* 1993). This was further evidence in support of a role for TGFβ in regulation of the ductal tree. *In vitro* studies on mammary epithelial cells treated with exogenous TGFβ resulted in the induction of an epithelial to fibroblastic transdifferentiation (Miettinen *et al.,* 1994). The effect observed was reversible and the change in cell morphology correlated to a loss of epithelial markers and an increase in expression of mesenchymal markers. Silberstein and colleagues (1992) observed that the periductal ECM acts as a TGFβ1 reservoir. Their studies revealed that TGFβ1 was acquired, maintained, and lost from this compartment during ductal growth and morphogenesis.

1.3.2.2 Inhibins and Activins

The inhibins and activins are also members of the TGFβ superfamily. To date three types of activins, activin A (βAβA), activin B (βBβB), and activin AB (βAβB) and two types of inhibins, βA (inhibin A) or βB (inhibin B) have been described. Activins and inhibins were first identified as gonadal factors which influence the production of follicle stimulating hormone in the pituitary.

Subsequently they have been shown to play roles in neuroendocrine regulation and also modulate luteotrophic hormone, growth hormone and adrenocorticotrophic hormone production. Mice deficient in the inhibin βB gene have no activin B or AB activity as well as a loss of inhibin B activity. These mice show retardation of ductal elongation and alveolar morphogenesis during puberty and pregnancy respectively (Robinson and Henninghausen, 1997). Mammary transplantation experiments have revealed this defect in development to be linked to the stroma. An abnormal morphology of TEBs was observed in the βB mice which implied the defect was due to a disruption in the balance between cell proliferation and cell death in the TEB. Although the βB deficient mice contain less mammary tissue than their wild type siblings, the alveolar epithelial cells displayed a differentiated phenotype compatible with milk protein gene transcription. The expression of WAP and β-casein during late pregnancy and lactation was similar in the βB deficient mice to that seen in the wild type littermates (Robinson and Henninghausen, 1997). It was proposed that these proteins have a paracrine mode of action on the mammary gland and this was further supported by observations in mice carrying a βA mutation. These mice have malformations in the secondary palates and an absence of teeth and whiskers, all of which depend on epithelial-mesenchymal interactions (Matzuk *et al.,* 1995). The exact mechanism through which inhibins and activins exert their effect on the mammary gland is not yet clear but there is some suggestion that they may be local mediators of hormonal signals.

1.3.2.3 Transforming Growth Factor Alpha and Epidermal Growth Factor

Transforming growth factor α (TGFα) has also been implicated in the growth of the mammary gland. TGFα is a secreted growth factor that binds to the same receptor as epidermal growth factor (EGF), and it acts as a potent mitogen for several cell types. TGFα promotes the proliferation and formation of lobuloalveolar structures in the mammary gland. Dickson and colleagues (1986) suggested that TGFα may be involved in local control of the growth and development of the mammary gland by acting as an intermediate in the action of

steroid hormones. The EGF-like growth factors and receptors are predominantly associated with breast cancer e.g. elevated levels of TGFα is seen in 70% of all breast cancers. Transgenic mice expressing mutant TGFα were observed to be fertile but did not lactate sufficiently to support a litter. Adult virgin mice displayed alveolar gland hyperplasia. Pregnant transgenics showed a marked proliferation of stromal cells and an increase in alveolar secretion when compared to wild type littermates. These mutant mice subsequently developed adenocarcinomas following multiple pregnancies (Matsui *et al.,* 1990). Mating of the TFGα and TGFβ transgenic mice revealed that these growth factors oppose the action of each other during mammary gland development. The effects that EGF exerts on mammary gland development have been studied using EGF containing pellets. Implantation into the mammary gland of ovariectomised mice revealed that EGF binding was restricted to the cap cells of the end buds, the ductal myoepithelium and the stroma surrounding the ducts and end buds (Coleman *et al.,* 1988). This resulted in end bud stimulation and the growth effects of EGF were observed to be synergistic when combined with TGFα (Vonderhaar *et al.,* 1987).

1.3.2.4 Colony Stimulating Factor-1

Colony stimulating factor 1 (CSF1) is a homodimeric growth factor originally identified in the regulation of proliferation, survival and differentiation of mononuclear phagocytes. It also plays an additional role in maternal-fetal interactions during pregnancy. A natural mutant, *op* (osteopetrosis) exists in the gene encoding CSF1. The major phenotype of these mice is the absence of functional osteoclasts and a failure in tooth eruption, but lactation is also severely compromised. Reduced ductal growth during pregnancy was observed together with the precocious development of lobules. An absence of milk secretion was also observed in the *op* mice (Pollard and Henninghausen, 1994). These mice revealed a previously unsuspected role for CSF1 in mammary development. CSF1 was observed to play a role in the regulation of branching morphogenesis during mid pregnancy and also be involved in the switch to lactation. The precise mechansim

by which CSF1 regulates these functions is presently unknown. It is unclear whether CSF1 exerts its effects within the mammary gland by directly affecting alveolar epithelial cell growth, or whether CSF1 affects gland development through the macrophages resident in the mammary gland. A third possibility exists that CSF1 affects another tissue, which produces a hormone or growth factor that acts to regulate mammary gland development e.g. the placental trophoblasts. This latter explanation fits best with the observation that exogenous administration of CSF1 to *op/op* mice cannot restore the lactation defect. The very high concentrations of CSF1 found in the utero-placental unit during pregnancy cannot be mimicked by systemic administration.

1.3.2.5 Insulin-like Growth Factor

Insulin-like growth factors (IGF) have been observed to play a role in gland development. Overexpression of IGF1 and the IGF binding protein 3 (IGFBP3) during late pregnancy and lactation was observed to have no effect on gland development and lactation. In contrast to this there was a delayed involution in these animals. Extensive lobulo-alveolar units remained in these mice and apoptotic cells were rare within these structures (Neushwander *et al.,* 1996). These results demonstrated a role for IGF1 and IGFBP3 in the involution process in the mammary gland. Overexpression studies with IGF2 were observed to generate mammary adenocarcinomas (Bates *et al.,* 1995).

1.3.3 Cell Cycle Regulation of Mammary Gland Development

A number of proteins involved in the control of the cell cycle have been demonstrated to be elevated in a high proportion of human malagnancies. This has directed interest to the effects of deletion or overexpression of these proteins within mammary glands.

1.3.3.1 Cyclin A

Transgenic mice overexpressing a non-degradable form of cyclin A during pregnancy and lactation have been produced. Cyclin A is a protein that functions during S phase of the cell cycle. These mice appeared to lactate normally and have no obvious defect in lobuloalveolar or ductal development, but they displayed an increased rate of apoptosis and nuclear abnormalities including karyomegaly and multinucleation. In contrast to this mice overexpressing the cyclin dependent kinase 2 (cdk2), which functions with cyclin A during S phase, were observed to have normal mammary glands. When these mice were mated and double transgenic animals produced, a more pronounced phenotype was observed than with either alone. The female mice displayed nuclear abnormalities, similar to the cyclin A transgenics but they showed an increased apoptosis. The apoptosis rate observed was four fold higher than that observed when mice overexpress cyclin A alone. These double transgenics had an impaired lobuloalveolar development and occasionally displayed lactational problems suggesting that cyclin A and cdk2 act in synergy to regulate mammary gland development during pregnancy (Bortner and Rosenberg, 1995).

1.3.3.2 Cyclin E

The D and E type cyclins are involved in regulating the progression of the G1 phase of the cell cycle. Alteration of cyclin E expression has been associated with breast cancers. Overexpression of cyclin E in the developing secretory epithelium of the gland results in a hyperplastic lactating gland (Bortner and Rosenberg, 1997). Ten percent of these transgenic mice further developed mammary adenocarcinomas suggesting deregulation of this protein can indeed induce neoplastic transformation of the mammary gland.

1.3.3.3 Cyclin D

Aberrant expression of cyclin D1, like cyclin E, has been associated with

breast cancer as well as a variety of other human malignancies. Cyclin D1 knockout mice develop to term but have reduced body size and a reduced viability. They also display neurological impairment and a retinal defect. Of the few homozygous mice that survive to adulthood, these develop mammary tissue that has the capacity to fully differentiate but these females are unable to nurse their pups due to a defect in pregnancy associated mammary proliferation. The phenotype displayed is a dramatic impairment of the expansion of the mammary epithelium, in particular the development of alveolar lobules in virtually absent in these mutant mice (Sicinski *et al.,* 1995). It is well documented that the proliferation observed in early pregnancy is a steroid driven event and the results obtained from the cyclin D1 knockout mice have suggested that this event may be driven by cyclin D1.

1.3.3.4 Tumour Suppressor Protein p53

The tumour suppressor protein p53 is implicated as a mediator of apoptosis in a wide range of tissues. It is involved in the cellular response to DNA damage. p53 functions to initiate a cell cycle arrest during which the cell can repair the damaged DNA and re-enter the cycle (Lane, 1992), or if the damage is excessive p53 can mediate an apoptotic response. Although this pathway is functional in the mammary gland the apoptotic response observed during involution was shown to be p53 independent (Li *et al.,* 1996). Knockout mice for p53 displayed no difference in involution when compared to control mice. These results suggested that apoptosis of the mammary alveolar cells and the collapse of lobulo-alveolar structures progressed independently of p53 function.

1.3.3.5 Temperature Sensitive Simian Virus 40 Antigen

Transgenic mice expressing the temperature sensitive simian virus (SV40) Tag in the mammary gland have been produced. Tag binds to p53 and the retinoblastoma protein (pRb) thereby inactivating them. As both of these proteins have a critical function in regulating the cell cycle their effect on mammary development was analysed using the SV40 Tag mouse. These mice were unable to

nurse their young at the first pregnancy due to an impairment in alveolar development. As a result the fat pad of these mice was not completely filled during pregnancy. An increase in apoptosis (5% compared to 0.2% in wild-type mice) was observed during late pregnancy in these transgenics and the mice did not develop mammary tumours until they had progressed through 3 to 5 pregnancies (Li *et al.*, 1996). These studies demonstrated that p53-independent apoptotic pathways exist within the mammary gland and that activity of the pRb protein is a pre-requisite for a functional mammary gland.

1.3.4 Fatty Acid Regulation of Gland Development

1.3.4.1 Adipocytes

The importance of epithelial-mesenchymal interactions in the mammary gland have been displayed in mice carrying a deletion in either the TGFβ or inhibin gene. Adipose tissue has also been demonstrated to be of prime importance in the regulation of epithelial cell growth. *In vitro* coculture systems have shown that extraparenchymal adipose tissue produced both substrate and soluble factors which had the capability to stimulate epithelial cell growth. When mammary epithelial cells were cultured with adipocytes a duct-like morphogenesis occurred along with the formation of a basement membrane (Wiens *et al.*, 1987). These cells were able to functionally differentiate when grown in the absence of collagen gels. The role of lipid stores in fat cells was examined further and revealed that unsaturated fatty acids, linoleic acid in particular, could stimulate the proliferation of mammary cells. Saturated fatty acids in contrast were inhibitory to growth (Wichs *et al.*, 1979). Linoleic acid was further reported to stimulate growth only in synergism with another growth promoting factor, such as EGF. Kidwell and colleagues (1982) proposed that a hormonal mechanism was driving this fat cell stimulation. It was hypothesised that prolactin could cause fatty acid release from the fat cells promoting fatty acid uptake by the epithelium, which itself may promote mammary

epithelial tree growth. What is clear from these studies is that fat cells have the capability of exerting a regulatory influence on the growth potential of the epithelial component of the mammary gland.

1.3.5 Extracellular Matrix Regulation of Mammary Gland Development

The ECM is an important component of the cellular environment. It is composed of glycoproteins, proteoglycans and glycosaminoglycans that are secreted and assembled locally. These organise into a network to which cells adhere. The mammary gland epithelial and mesenchymal components are separated by a basement membrane (BM). The BM is the major structure of the ECM with which the mammary epithelial cells have direct physical contact. It consists of four ubiquitous components: laminin, entactin, type IV collagen and perlecan, a heparin sulfate proteoglycan. The other ECM components of the mammary gland are located predominantly in the stroma and include type I and III collagens, and ECM glycoproteins such as fibronectin (Lochter and Bissell, 1995). The BM functions in controlling normal development by positively regulating genes necessary for morphogenesis and differentiation and negatively regulating expression of genes which would compromise tissue specific function. Both laminin, required for milk protein production (Streuli *et al.,* 1991), and collagen, essential for epithelial morphogenesis (Lee *et al.,* 1985) have positive effects on mammary gland development. ECM binding by growth factors e.g. TGFβ, can function as a local store of growth factor that can persist after the growth factor production has ceased. Some ECM proteins contain intrinsic growth factor activity. This is achieved by repeated EGF-like sequences. These include the ECM protein laminin, the activity of which is localised to the EGF-like domain. Presently it is unclear whether laminin acts through the EGF receptor as well as integrin receptors. The effects that ECM proteins have on epithelial cells are mediated by specific receptors. The integrins are the best characterised of the ECM receptors and are heterodimeric proteins that are classified into subgroups according to their β subunit. The ligand

specificity of these receptors has been observed to be cell type specific e.g. the α1β1 integrin acts as a collagen receptor in platelets, but is both a collagen and laminin receptor in endothelial cells (Hynes, 1992). Most integrins are expressed by a variety of cell types, although some such as the β2 group have a more limited tissue distribution. Targeted disruption of integrin genes are generally lethal demonstrating that one integrin carries out an essential function than no other integrin provides.

1.3.5.1 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), are major regulators of ECM catabolism in mammalian cells. MMPs are generally secreted as inactive multidomain proenzymes, which are cleaved by a mechanism as yet unknown to form an active enzyme. The MMPs function predominantly during involution when major remodeling is required. Expression of MMPs remains low at day 3 after weaning, whereas TIMP1 was upregulated at this time shifting the proteolytic balance in favour of inhibition. At day 4 involution an upregulation of mRNA and protein levels of the protelytic enzymes gelatinase A, stromelysin1 and the serine proteinase urokinase-type plasminogen activator (uPA) has been observed. This correlated to a downregulation of the inhibitor TIMP1 which results in an active tissue remodeling and destruction of the basement membrane (Lund *et al.*, 1996). The expression of these ECM regulating proteins correlates with the two stages of involution observed within the mammary gland. The mammary gland also displays a significant amout of proteinase activity during virgin gland development and pregnancy, stages where ECM remodeling is required. Mice overexpressing stomelysin1 (SL1) show profound changes in mammary gland function. In virgin mice this results in a premature branching morphogenesis, differentiation and hyperproliferation. These mice are predisposed to forming mammary gland tumours. During pregnancy the epithelial cells undergo an unscheduled apoptosis. This is thought to be a consequence of SL1 initiating degradation of the basement

membrane. This degradation induces apoptosis by inducing expression of the caspase interleukin-1β converting enzyme (ICE), a protease involved in the terminal events during apoptosis. When SL1 is overexpressed during lactation the mammary glands contain smaller collapsed alveoli and resemble an early involution phenotype (Sympson *et al.*, 1994). These results suggested a role for SL-1 in regulating stromal-epithelial interactions in the gland. Transgenic mice overexpressing TIMP-1 showed a delay in alveolar development early in pregnancy. By late pregnancy no discernible difference was observed between TIMP-1 overexpressing and wild-type mice. Lactational development was similar to control glands but fatty infiltration was accelerated during involution (Alexander *et al.*, 1996). The involution process was itself slightly delayed in these mice. Crossing TIMP-1 transgenic mice with SL-1 transgenic mice was observed to block the premature involution observed during pregnancy with ectopic SL-1 expression.

1.3.5.2 Glucocorticoid Effects on Mammary Gland Development

Glucocorticoids are thought to act as survival factors in mammary epithelial cells. Hydrocortisone pellets implanted in the mammary gland have little effect on the first stage of involution but completely inhibit the second remodelling stage, despite being added 3 days following weaning. (Lund *et al.*, 1996). Dexamethasone, as well as progesterone, have been shown to inhibit involution in the mammary gland. The function of the transcription factor AP-1 was observed to be impaired when mammary glands were exposed to dexamethasone (Feng *et al.*, 1995). Many of the genes which are upregulated during involution e.g. SL-1, uPA, c-*jun* and sulphated glycoprotein-2 (SGP-2) have been shown to contain AP-1 response elements within their promoter regions suggesting that dexamethasone exerts its inhibitory effects on involution by preventing the expression of the necessary MMPs. These results suggest that *in vivo* gene expression of MMPs is under hormonal control. Manipulation of this class of proteins *in vivo* has demonstrated the requirement for a critical balance between the ECM-degrading proteinases and their inhibitors. This has revealed the role the ECM plays in the regulation of epithelial cell function and morphology during mammary gland

development and involution

1.4 Signalling Pathways in the Mammary Gland

1.4.1 Prolactin Signalling

The signalling pathway of prolactin was made accessible by the cloning of the prolactin receptor in 1988 (Boutin *et al.*, 1988). It is a transmembrane receptor which belongs to the cytokine receptor superfamily. Alternative spliced products exist resulting in receptors which have either long or short cytoplasmic tails. Both forms of this receptor can bind prolactin and dimerise activating the Janus Kinase2 (Jak2), Fyn, and mitogen activated protein (MAP) kinase. *In vitro* work has shown that only the long form of the prolactin receptor can activate the transcription of the milk protein gene, β-casein. (Das and Vonderhaar, 1995). Activation of Jak2, situated on the box region near the membrane of the prolactin receptor results in phosphorylation of two Stat proteins, Stat5a and Stat5b in the case of prolactin. Tyrosine phosphorylation of the Stat proteins results in dimerisation and translocation to the nucleus where they induce transcription of genes containing γ-interferon activation sites (GAS) [Figure 1.3].

Stat5a and Stat5b have a 96% similarity and display an identical pattern of expression during pregnancy and lactation. It was thus expected that Stat5a and Stat5b deficient mice would display similar phenotypes. Stat5a deficient mice were unable to lactate due to the failure of the gland to develop fully and to undergo functional differentiation during pregnancy (Lui *et al.*, 1997). Stat5b levels and phosphorylation in these mice were greatly reduced suggesting that Stat5b requires the presence of Stat5a for efficient phosphorylation. The mechanism underlying this event is not clear at present but it has been hypothesised that Stat5a may be

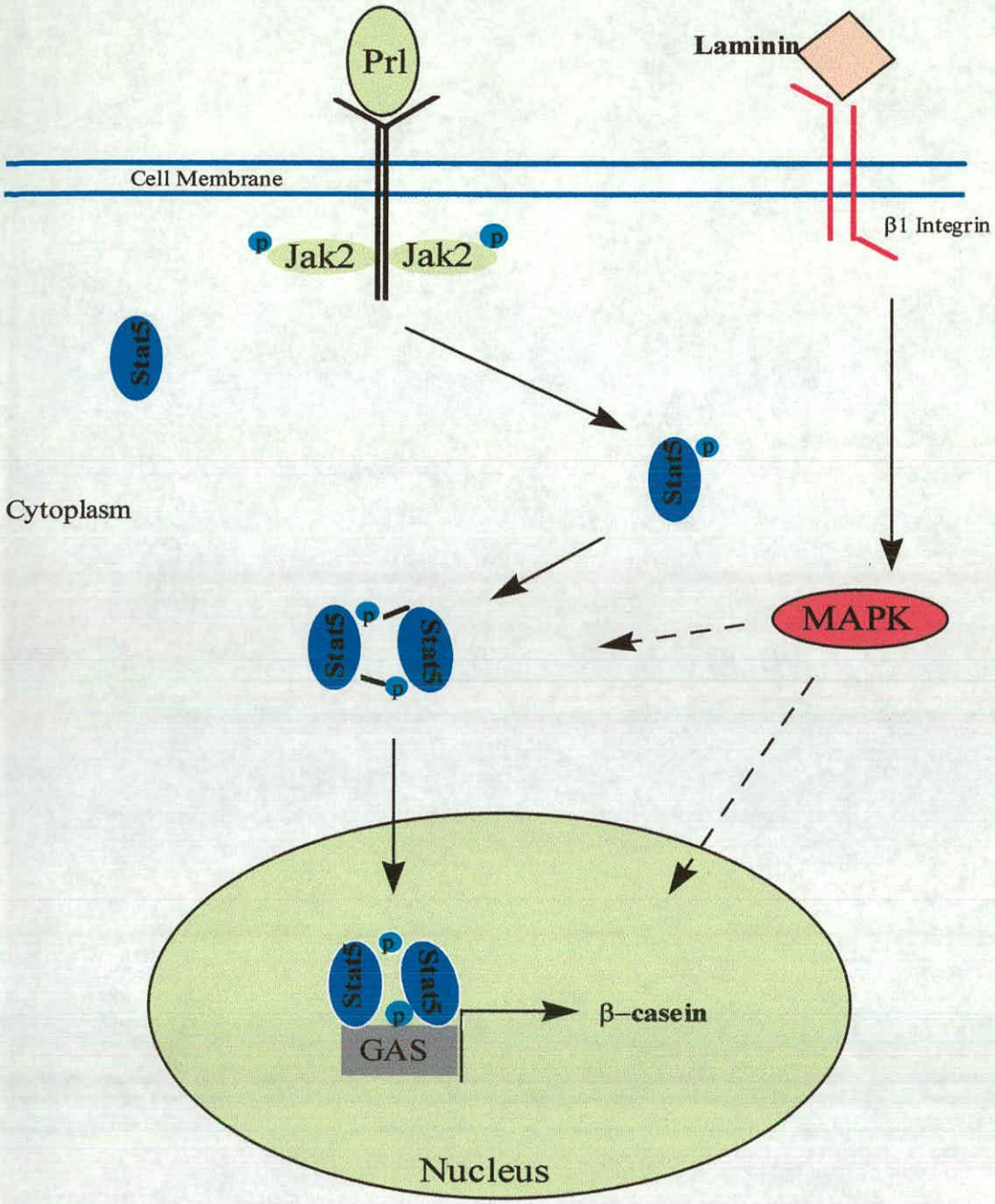

Figure 1.3 Prolactin Signalling Pathway. Prolactin binding to its receptor results in phosphorylation of Jak2, which in turn phosphorylates Stat5. This results in Stat5 dimerising and translocating to the nucleus where it binds γ-interferon sites (GAS) and activates transcription of its target genes e.g. milk protein genes. At present it is assumed that integrin signalling also interacts with this pathway but the exact signalling components are unknown.

necessary to achieve and maintain a state of cell differentiation, which itself may be a pre-requisite for full Stat5b activation. In contrast to this Stat5b deficient mice displayed a different phenotype where liver gene expression and growth rates were affected. The fertility in these mice was severely compromised (Udy *et al.*, 1997) but the mice which were able to mate maintained their pregnancies, delivered normal litters and could lactate. Both promoters of the milk protein genes of WAP and β-casein contain GAS sites, but only WAP gene expression was reduced in Stat5a deficient mice suggesting that the regulation of β-casein gene expression is controlled by other transcription factors. Although work from *in vitro* studies on the signalling pathways of Stat5a and Stat5b described them to be indistinguishable, *in vivo* these proteins may have distinct functions or conversely the relative amount of each protein may be the determining factor.

1.4.2 LAR Signalling

An additional signalling pathway that has been disrupted and shown to display a mammary phenotype is the LAR pathway. LAR is a protein tyrosine phosphatase (PTPase) which signals via the LAR receptor to function in regulating tyrosine phosphorylation through either cell-cell or cell-matrix interactions. LAR has a broad tissue distribution and its expression is regulated during pregnancy. Expression is maximal on day 16 gestation in wild type mice. LAR knockout mice have been observed to display no obvious phenotypes with the exception of a mammary gland abnormality. These mice are unable to produce milk due to an impaired terminal differentiation at late pregnancy. This failure to terminally differentiate results in a inability to switch to the lactation state and the mammary glands of these mice display a rapid involution postpartum (Schaapveld *et al.*, 1997). These observations would suggest that LAR mediated signalling plays an important role in mammary gland development and function.

1.5 Mammary Gland Overview

The development of the mammary gland requires the sequential activiation of a variety of hormones, growth factors, transcription factors, and ECM components at specific stages in gland development. This complement of factors, when activated correctly results in a functional gland expressing milk proteins in the lactation period following pregnancy. Most of the work that has lead to the assignment of gene function in gland development has arisen from transgenic or knockout mice. Although these mice were useful in detailing an absolute requirement for a gene product, they gave no information pertaining to the time window in which the particular gene was required. The introduction of new technologies allowing time specific, tissue specific gene deletion or inactivation systems e.g. Cre-mediated gene deletion systems (Wagner *et al.*, 1997, Lakso *et al.*, 1992, Pichel *et al.*, 1993) and tetracycline mediated gene expression systems (Gossen *et al.*, 1995) should provide new opportunities to examine specific windows of gene expression. The information available on mammary gland development to date may yet be revealed to be an oversimplified view of this developmental process and new technologies may further enhance our understanding of this unique organ.

The aim of this study was to use the technique of differential display in an attempt to identify genes that showed altered patterns of expression during pregnancy in the murine mammary gland. We chose to examine gene expression during pregnancy with particular relevance to specific stages of development e.g. day 5 gestation marks the peak of proliferation in the gland, day 10 gestation marks the onset of differentiation, and day 15 gestation marks terminal differentiation. As part of this work I identified a gene which functions as part of a protein degradation pathway. The following sections describe this pathway of protein degradation in detail. The revelance of proteolysis to mammary gland development will be discussed later.

1.6 Proteolytic Pathways Within the Cell

In the late 1930s Schoenheimer, using isotope tracer techniques, was the first to show that proteins within an animal are in a dynamic state, being continuously synthesised and degraded. The early progress in proteolysis research was hampered by technical hurdles but work in the last 20 years has demonstrated that the system responsible for the majority of intracellular degradation is the ubiquitin-proteasome system. The development of a cell-free lysate from rabbit reticulocytes in the 1970s allowed the biochemical dissection of this pathway to begin (reviewed in Hershko and Ciechanover, 1992). This initial work identified that ubiquitin conjugation of the substrate was an essential feature of degradation. At the same time the study of proteasomes was initiated but the connection with ubiquitin dependent proteolysis was not realised until 1987 when a protease capable of degrading ubiquitinated protein conjugates was purified (Hough *et al.*, 1987).

Within mammalian cells two distinct proteolytic pathways can be identified. Proteins which enter the cell from the extracellular environment e.g. receptor mediated endocytosed proteins, are degraded by lysosymes. Lysosomal degradation of intracellular proteins occurs only under conditions of stress. Although lysosomes are important degradative organelles most of their proteolysis is relatively non-specific. The vast majority of intracellular degradation that occurs is mediated by the ubiquitin-proteasome pathway. This complex is involved in the selective turnover of proteins under basal metabolic conditions. Other proteolytic enzymes exist within cells e.g. the ICE-like proteases, which are crucial for apoptosis, and the calpains. Although systems other than the proteasome exist evidence suggests that these secondary systems are limited in either their range of substrates or under the conditions in which they are functional.

Cells are often required to switch from one cellular state to another in response to environmental signals or as part of regulated developmental pathways. Switches of this manner require rapid dismantlement of existing regulatory

networks, which are often dependent on protein degradation. Selective proteolysis offers two main advantages over other possible control mechanisms. It is a fast process which enables a cell to rapidly reduce the level of a defined protein and it is also an irreversible process, ensuring complete loss of protein function with no chance of inappropriate reactivation. These features help explain why selective protein degradation plays a prime regulatory role in systems which rely on timing controls. The specificity of protein degradation must be extremely high as degradation of proteins at inappropriate times would have serious consequences to the cell.

1.6.1 The ubiquitin-proteasome pathway

Conjugation to ubiquitin is an essential step in the degradation of many short-lived eukaryotic proteins. The pathway for proteasome mediated degradation is shown in figure 1.4. Initially ubiquitin must be activated prior to addition to the substrate. Adenylation of ubiquitin is mediated through a ubiquitin activating enzyme, E1. This reaction generates a ubiquitin-E1 thiolester which is subsequently passed onto an ubiquitin-conjugating enzyme, E2. E2 enzymes function either alone or in conjunction with an ubiquitin-protein ligase, E3 to catalyse substrate ubiquitination. The ubiquitinated substrate is then subject to further rounds of ubiquitination, ubiquitin removal by deubiquitinating enzymes or degradation by a multicatalytic protease, the 26S proteasome. The proteasome functions to degrade ubiquitinated proteins to short peptides, most of which are rapidly hydrolysed to amino acids by cytosolic endopeptidases. A portion of the peptides generated by the proteasome are presented to the immune system on the major histocompatibility complex (MHC) class I molecule.

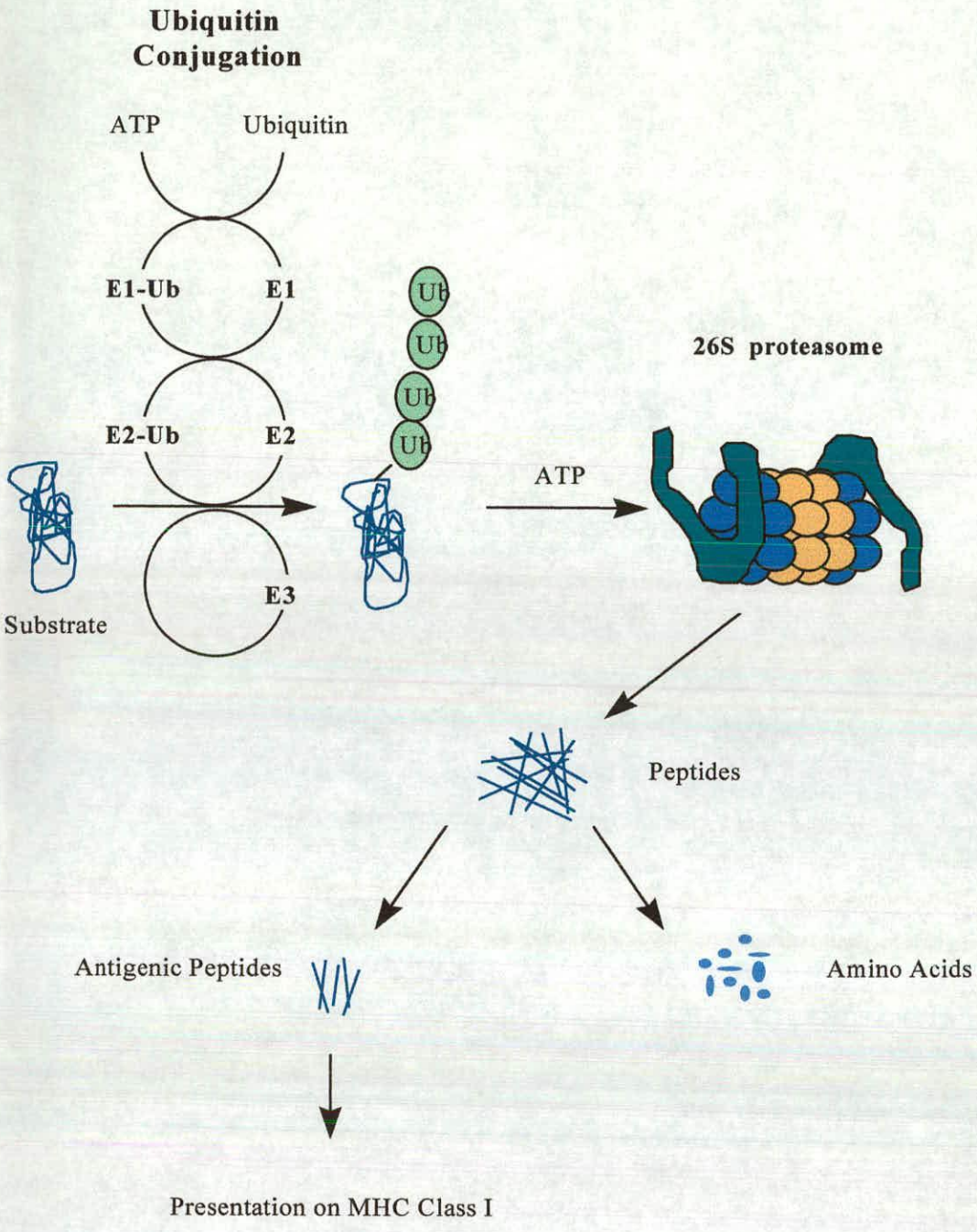

Figure 1.4 Ubiquitin-Proteasome Degradation Pathway. Native proteins are ubiquitinated by a three enzyme cascade (E1-E3), which results in ubiquitin linkage to a lysine residue on the protein. Further ubiquitin molecules are linked to the first one, forming a chain of ubiquitin on the protein. These chains are recognised by the 26S proteasome, where the ubiquitin bonds are cleaved by an isopeptidase and the substrate is unfolded by an ATPase, all located in the 19S cap or regulatory complex. The degraded peptides generated by this complex are either degraded into their constituent amino acids or are presented as antigenic peptides on MHC class I molecules.

1.6.2 Protein Ubiquitination

The prime step which determines whether a particular protein will be degraded by the proteasome is its modification by ubiquitin. This reaction is catalysed by a series of enzymes. Although a wealth of information has been made available recently on this proteolytic system, the precise mechanism by which ubiquitin transfer is achieved remains poorly understood. The ubiquitin-proteasome system is a highly conserved pathway that is very similar in species as distant as yeast and mammals. For these reasons I shall describe the system utilised by the yeast *Saccharyomyces cerevisiae* as the genome of this organism is now fully sequenced.

1.6.2.1 Ubiquitin Activating Enzyme (E1)

The initial stage in targeting a specific protein for degradation is the activation of ubiquitin. Ubiquitin is a 76 amino acid protein which is highly conserved and present in all eukaryotes. It has been shown to be essential for cell viability (Finley *et al.,* 1994). Activation of ubiquitin involves the formation of an E1-ubiquitin thiolester. E1 has been purified from a variety of sources and was demonstrated to exist as a homodimer of 210kDa. Two further E1 enzymes have been cloned in yeast, wheat and humans (Table 1.1). These genes have been shown to highly conserved in evolution e.g. the human and yeast proteins are 53% identical (Hershko and Ciechanover, 1992). E1 proteins contain two short stretches of amino acids which resemble a nuclear localisation signal, in addition to regions which are characteristic of nucleotide binding proteins. Conditionally lethal alleles of E1-encoding genes have been isolated in mammalian cell lines, and a gene encoding yeast E1, UBA1 was found to be essential for growth (Ciechanover, 1994), whereas another, UBA2 is required for viability (Dohmen *et al.,* 1995). The hypothesis held with regard to the three distinct E1 enzymes suggests that each of these enzymes may prefer a specific set of E2 enzymes or that they may reside in a particular cellular compartment thus the requirement for more than one enzyme exists.

Protein	Size (kDa)	Function
Uba1	114	Essential for viability. Forms thiolester with ubiquitin
Uba2	71	Essential for viability. Predominantly nuclear in expression
Uba3	33	Function unknown.

Table 1.1 *S. cerevisiae* E1 enzymes.

1.6.2.2 Ubiquitin Conjugating Enzyme (E2)

Ubiquitin is transferred from the E1 enzyme to an E2 enzyme by the process of transthiolation. These proteins subsequently mediate the transfer of ubiquitin to the substrate protein with or without the involvement of an ubiquitin-protein ligase (E3). Examination of rabbit reticulocyte lysates revealed the presence of multiple E2 isoforms and genetic analysis has confirmed that these proteins are encoded by a family of related genes. In yeast 13 E2 related enzymes have been identified and these are listed in Table 1.2. Structurally these proteins all contain a core of approximately 150 amino acids which display 35% sequence homology. The active site cysteine residue that is required for the formation of the thiol ester with ubiquitin is found in this domain. Some of the E2 enzymes that have been identified are small 14-18kDa proteins that consist almost exclusively of this conserved domain. Other enzymes have C-terminal extensions which are either neutral e.g. Ubc1, or highly acidic e.g. Ubc4, Ubc5 and Ubc7. Mutational analysis of E2 enzymes have shown a range of phenotypes exist (Table 1.2) suggesting that these enzymes have different functions or that they display distinct substrate specificities. Multiple E2 enzymes have been shown to be required for the degradation of one substrate. The best example of this is the yeast MATα2 transcriptional repressor. Four E2 enzymes are involved in the degradation of this

protein. Ubc6 and Ubc7 have been shown to complex and function in one pathway for α2 degradation (Chen *et al.*, 1993). Mutants lacking either one of these enzymes display distinct phenotypes suggesting that these proteins can act independently in the degradation of other substrates. Analysis of *in vitro* protein ubiquitination has suggested that there may be some redundancy within this group of enzymes. Any one of three different E2 enzymes can ubiquitinate the tumour suppressor protein p53 *in vitro*. During mitosis cyclins are also ubiquitinated by a number of E2 enzymes, the only difference between these proteins being that some enzymes produce fewer ubiquitin-conjugated molecules than others. Whether this overlay in function occurs *in vivo* is yet to be elucidated.

1.6.2.3 Ubiquitin-Protein Ligase (E3)

The component of the ubiquitin conjugation system that is most directly involved in substrate recognition is E3. Two species of E3, E3α and E3β were initially isolated from reticulocyte extracts. E3α is the most thoroughly studied of these enzymes and it was first described as an enzyme necessary, in addition to E1 and E2 for the ligation of ubiquitin to specific proteins (Hershko *et al.*, 1983). E3 enzymes promote the addition of multiple ubiqutins to the substrate protein and are also involved in the formation of polyubiquitin chains. Purification of the enzyme revealed that E3α existed as a homodimer which contained sites which recognised N-terminal residues of the substrate (the N-end rule, which will be discussed in section 1.6.3.1). The enzyme has two distinct sites, one which recognises basic residues whilst the other recognises hydrophobic N-terminal residues (Reiss *et al.*, 1988). The signals responsible for the binding of acidic amino termini to E3α remain unclear as yet. This class of enzymes also contains a site which binds E2 enzymes in addition to ubiquitin. It is hypothesised that the ubiqutin site might be required for the addition of multiple ubiquitin chains to the

Protein	Size (kDa)	Function
Ubc1	24	Essential in the absence of Ubc4 and Ubc5. Endocytosis of membrane proteins
Ubc2/Rad6	20	DNA Repair, induced mutagenesis, and N-end rule pathway
Ubc3/Cdc34	34	Essential for viability, G1/S phase cell cycle progression
Ubc4	16	92% identical to Ubc5. Functional overlap with Ubc5
Ubc5	16	Degradation of abnormal protein, MATα2 and ubiquitin-fusion proteins
Ubc6/Doa2	28	Degradation of MATα2. Localised to endoplasmic reticulum/nuclear envelope.
Ubc7	18	Functional overlap with Ubc6.
Ubc8	25	No phenotype found in null mutant.
Ubc9	18	Essential for viability G2/Mitosis cell cycle degradation
Ubc10/Pas2	21	Peroxisome biogenesis
Ubc11	17	Function unknown
Ubc12	21	Function unknown
Ubc13	18	Function unknown

Table 1.2 *S. cerevisiae* E2 Enzymes. The human homologues of these enzymes are named Ubch.

substrate. The yeast homolgues of these proteins are listed in Table 1.3. Although the properties of E3α described above have been attributed to this class of enzyme, distinct groups of E3 ligases exist. Studies on p53 degradation by the human papilloma virus protein E6 have revealed a new E3 protein, E6 associated protein (E6-AP). E6-AP, together with E6 binds p53 and stimulates its degradation. The E6-AP/E6 complex has not been shown to bind any E2 enzymes. Despite this E6-AP can transfer ubiquitin from E2 to the substrate protein suggesting that it must at least transiently interact with E2 enzymes (Scheffner *et al.,* 1995). The C-terminal 350 residues of E6-AP show sequence similarity to a number of proteins in a range of organisms and has been named the HECT domain (homologous to E6-AP carboxyl-terminus). The HECT domain contains an absolute conserved cysteine residue essential for ubiquitin thoiolester formation and substrate ubiquitination. Five different HECT domain proteins have been identified in yeast, some of which are essential for viability. Ubr2 has been identified in sequence as similar to Ubr1 (22% identical) although to date no data has been published on Ubr2. The cyclosome/anaphase promoting complex (APC) has properties that suggest it may function as an E3. APC is composed of three subunits, none of which have homology to any known E3s, and is involved in catalysing the degradation of cyclin B in control of cell cycle progression. Although to date there is no clear mechanism defining the function of an E3 enzyme it seems highly probable that a number of additional E3-like factors will be identified in the near future.

The successive action of the E1 to E3 enzymes results in the formation of isopeptide bonds between the ε-amino groups of lysine residues in the substrate and the carboxyl-terminal glycine residues of ubiquitin. Branched polyubiquitin chains are formed by the sequential addition of monomers of ubiquitin to lysine 48 of the substrate bound ubiquitin (reviewed in Ciechanover, 1994).

Protein	Size (kDa)	Function
Ubr1-Related		
Ubr1/Ptr1	225	Degradation of N-end rule substrates
Ubr2	217	Not determined
Hect-Domain Proteins		
Rsp5/Npi1	92	Essential for viability. Forms a thiolester with ubiquitin
Ufd4	168	Degradation of ubiquitin fusion proteins
Tom1	374	Essential for viability. Trigger Of Mitosis
Hct4	103	Not determined
Hct5	168	Not determined

Table 1.3 *S. cerevisiae* E3-like Proteins. Potential E3-like proteins that are less well characterised are not listed. Additional E3-like proteins exist in humans e.g. E6-AP, and APC.

1.6.2.4 Deubiquitinating Enzymes

The vast majority of the research on the ubiquitin conjugation system has focused on the enzymes which attach ubiquitin to proteins but more recently the regulatory events that control protein deubiquitination has become of prime importance. An essential step in the function of this system is the recycling of free ubiquitin. Ubiquitin is cleaved from substrates by a group of enzymes known as deubiquitinating enzymes, ubiquitin carboxyl-terminal hydrolases, or ubiquitin isopeptidases. *In vitro* studies have revealed that these enzymes can be classified into two major groups; small proteins which cleave ubiquitin from peptides and a group of larger proteins which can generally cleave ubiquitin from a range of protein substrates. The majority of work has focused on the later class of protein, which are also known as ubiquitin specific proteases (Ubps). The Ubp family of enzymes is large, with 16 enzymes identified in *S. cerevisiae* (Table 1.4). The family is extremely divergent but all members of the family contain a short

consensus sequence. This sequence is composed of cysteine (Cys) and histidine (His) boxes which are thought to form the active site of the enzyme. The high number of deubiquitinating enzymes identified has suggested that these enzymes can differentially control protein turnover rates. The possibility also exists that Ubps can display a degree of substrate specificity and that they may be expressed in different cellular compartments. Yeast mutants in a range of Ubps have been observed to display very little phenotypic aberrations suggesting that a degree of redundancy exists in this class of enzymes. Evidence in support of a substrate specific modulation of degradation was gained from studies of a Ubp in *Drosophila*, the *fat facets* (*faf*) gene. Huang and colleagues (1995) have shown the *faf* gene was required for normal eye development, and that embryos from homozygous mutant mothers died at an early stage in development. Mutations in the conserved Cys and His boxes in Faf show identical effects as those flies containing null mutations. These studies have suggested that it is the deubiquitinating activity of Faf that is critical to its biological function. Huang has shown that Faf functions to reverse the ubiquitination of specific proteins thus preventing their degradation by the proteasome. Mutations in components of the 20S proteasome were observed to reverse the defect seen in *faf* flies. The murine homologue of this gene (Fam for *fat facets* in mouse) has been demonstrated to be widely expressed during postimplantation development. This suggested that Fam was not associated with a single developmental event (Wood *et al.*, 1997).

It is hypothesised that Ubps function to alter the balance between the rates of ubiquitination and deubiquitination and thus act as negative regulators of the ubiquitin system. Ubps can function in several ways to enhance protein ubiquitination or degradation. These enzymes are required to generate ubiquitin monomers. Ubiquitin is synthesised as a precursor protein which requires the action of Ubps to remove C-terminal amino acids. Activated ubiquitin can itself form adducts with intracellular nucleophiles e.g. amines or glutathione. This process could consume the pool of free ubiquitin very rapidly if they were not immediately broken down. Long ubiquitin chains are assembled on intracellular proteins and in some situations this can result in the inappropriate tagging of a

protein. Ubps in this system can function to prevent the inappropriate degradation of proteins resulting in a highly selective degradation system. Proteasomes and E3 enzymes can bind ubiquitin chains and these need to be quickly disassembled to prevent inhibition of proteasomes. A variety of evidence now exists for a number of different types of deubiquitinating enzymes. An editing ubiquitin isopeptidase has been described within the 19S cap complex of the proteasome. The enzyme is though to function in two manners: to shorten polyubiquitin chains to facilitate translocation of the substrate towards the proteolytically active sites of the proteasome, and to deubiquitinate the substrate which may be essential for the complete degradation of the substrate (Lam *et al.*, 1997). This editing Ubp may also function to release partially degraded proteins from the complex e.g. for the proteolytically processed NFκB precursor p105. Mammalian isopeptidase T (isoT), which is the functional homologue of yeast Ubp14, exclusively acts on unanchored ubiquitin chains. Yeast mutants lacking Ubp14 have defects in ubiquitin mediated degradation of a number of substrates and show an accumulation of unanchored ubiquitin oligomers (Hadari *et al.*, 1992). The phenotype displayed by the yeast Ubp14 mutant could be reversed by expression of human isoT. The observation that ubiquitinated species accumulated in yeast cells carrying certain Ubp mutants e.g Ubp14 and Ubp4 was of surprise initially. It was expected that these mutations would show no alteration in degradation due to the high number of Ubp enzymes. These results suggested that the Ubp enzymes may exert a very high substrate specificity or as an alternative theory that these ubiquitinated species accumulate in cellular compartments where they are inaccessible to the majority of Ubps. Work in mammalian systems have described a regulatory function for Ubps. The human *tre*-2 oncogene (yeast homologue Ubp4) is an inactive form of a deubiquiting enzyme that is tumorigenic. The deregulation of growth observed in *tre*-2 mutant cells may be attributed to a failure to degrade short-lived peptides which are essential for cell proliferation (Papa and Hochstrasser, 1993). DUB-1 is a murine Ubp which is an erythroid cell specific, immediate early gene induced by interleukin 3 (IL-3). Mutants in DUB-1 show a G1 cell cycle arrest suggesting a growth regulatory role for this Ubp (Zhu *et al.*, 1996). A vast number of deubiquitinating enzymes have been described recently and our current knowledge of these enzymes and their

Protein	Size (kDa)	Function
Ubp1	93	Cleaves ubiquitin protein fusions. No phenotype reported for mutant
Ubp2	145	Cleaves ubiquitin protein fusions. No phenotype reported for mutant
Ubp3	102	Cleaves ubiquitin protein fusions. No phenotype reported for mutant
Ubp4/Doa4	105	Degradation of ubiquitin system substrates. Maintenance of ubiquitin pools. Mutant has slight growth defect.
Ubp5	92	Cleaves ubiquitin protein fusions. No phenotype reported for mutant
Ubp6	57	Not determined
Ubp7	123	Cleaves ubiquitin protein fusions. Mutant is viable
Ubp8	54	Mutant has a slight growth defect
Ubp9	86	Cleaves ubiquitin protein fusions. Mutant is viable
Ubp10/Str4	90	Mutant has a defect in chromatin-mediated gene silencing
Ubp11	83	Not determined
Ubp12	143	Not determined
Ubp13	84	Not determined
Ubp14	91	Degradation of MATα2 and other proteins. Cleaves ubiquitin oligomers with free C-terminus *in vivo* and *in vitro*
Ubp15	143	Mutant has slight growth defect
Ubp16	57	Not determined
Yuh1	26	Cleaves ubiquitin from small adducts. No phenotype reported for mutant

Table 1.4 *S. cerevisiae* Deubiquitinating Enzymes.

mode of action are limiting. The roles these enzymes play in the processes of growth and development may become clearer in subsequent years.

1.6.3 Regulation of Protein Ubiquitination

A central problem to this system is exactly what structural features of proteins are recognised by the ubiquitin system. Early in vitro work suggested that E3 ligases directly recognised the substrate and functioned as an adapter molecule (reviewed in Hershko and Ciechanover, 1992). As some E3s e.g. E6-AP do not form tight complexes with their specific E2s, then this theory seemed highly unlikely. It has also been demonstrated that a small number of E2 enzymes can function alone to directly ubiquitinate a substrate protein in the absence of an E3 enzyme. The ubiquitination complex can either assemble ubiquitin chains directly onto the substrate by repeated addition of ubiquitin or transfer a preassembled chain onto the target protein (reviewed in Hochstrasser, 1996). Both of these processes have been observed in vitro but the significance that this may have on the rate of substrate degradation is presently unclear. In vitro monoubiquitination has been observed to be sufficient for substrate degradation but it is thought that proteasome prior to deubiquitination. This situation may exist if proteins had different susceptibilities to deubiquitination, which at present is unknown. Ubiquitin chains attached to a substrate protein may act as a tether for proteins that may be more difficult to denature than normal. Thus multiubiquitination may serve to bind substrates to the proteasome long enough to be fully denatured prior to deubiquitination.

Proteins that are substrates of the ubiquitin-proteasome degradation pathway have been observed to contain sequence elements that target them for rapid turnover. These following data was obtained using studies where these sequence specific regions were placed on a different protein.

catalytic complex. This can be joined on either end by a 19S regulatory complex or an 11S-activator complex. This results in two different types of proteasome present within the cell, a 20S-19S complex (also called the 26S proteasome) and an 20S-11S complex. The following sections describe the structure and function of each of the three possible components of an active proteasome.

1.6.4.1 The 20S Proteasome

The 20S core proteasome contains all of the catalytic activity of the 26S proteasome. It was originally identified by electron microscopy as a ring-like structure which was interpreted as a side-on view of a cylindrical particle (150Å in height and 110Å in diameter) composed of four stacked rings, with each ring being composed of 7 subunits. These particles were shown to be 700kDa protein complexes composed of 14 subunits with sizes ranging from 19 to 36kDa. The vast majority of the early research on proteasome structure was obtained from studies using the archeon *Thermoplasma acidophilum*. The *T. acidophilum* proteasome has been observed to be structurally highly similar to proteasomes from higher species. This proteasome is composed of 4 stacked rings that are derived from two related subunit types, α and β. The α subunits form the outer two heptameric rings and the β subunits the inner rings giving a α7β7β7α7 structure. In yeast there are 14 genes which encode subunits of the 20S proteasome and these can be grouped into seven α-type and seven β-type subunits (Table1.5 and Table 1.6). All of these subunits are closely related in sequence to the α or β subunits in Thermoplasma. Sequence similarity also exists between the α and β subunits suggesting that all proteasome subunits were derived from a single ancestral gene early in evolution which has been confirmed by phylogenetic analysis (Hughes, 1997).

Of the seven α-type subunits identified all but one are required for viability

in yeast. These subunits are characterised by three conserved α-boxes, which are located within the N-terminal two-thirds of the proteins. A highly variable C-terminus is the region that confers specificity to the seven different α subunits. Several of these subunits contain sequences which resemble tyrosine kinase or cAMP/cGMP dependent kinase phosphorylation sites. Four of the mammalian α-type subunits have been observed to contain nuclear localisation signals (NLS), and work performed on a *Drosophila* α subunit has confirmed that these NLS are functional (Knuehl *et al.*, 1996). The mode of nuclear localisation is unclear at present but it has been hypothesised that regulation of the phosphorylation state of the α subunits may allow the NLS to become accessible when the proteasome is required to function in a nuclear capacity. Mason and co-workers (1996) have identified two phosphorylated α subunits in rat proteasome adding support to this hypothesis. It was shown that the elimination of a NLS in one of the α subunits could not be compensated for by the activity of another subunit. Although the NLS is functional it was not seen to be able to promote complete nuclear translocation suggesting that the subunits are subject to an additional control mechanism to determine cellular location. Two subunits (Pre 6 and Pre 10) have been observed to contain KEKE motifs suggesting that these proteins function in interacting with other proteins. As these two α subunits are neighbours it has been suggested that they may form the point of association with the regulatory complex (Kopp *et al.*, 1997).

The β-type subunits differ from the α-type subunits as they are less conserved between each other. They also lack the conserved N-terminal α-box domain found in the α subunits. In mammals three additional non-essential β subunits can be found. LMP2, LMP7, and MECL1 can replace constitutive components of the proteasome (X, Y, and Z subunits) when stimulated by the cytokine interferon-γ (IFN-γ). Mice carrying deletions of the LMP2 or LMP7 genes show a mild phenotype with altered cleavage properties of the 20S proteasome and an impairment in MHC Class I antigen presentation (Stohwasser *et al.*, 1996). Five

Protein	Size (kDa)	Function
Sc11	28	Unknown
Doa5/Pup2	29	Required for sporulation, degradation of ubiquitinated proteins
Pre5	26	Unknown
Pre6	28	Unknown
Pre8	27	Point mutation can be suppressed by hsp70 overexpression
Pre9	29	Only non-essential 20S proteasome subunit
Pre10	32	Unknown

Table 1.5 *S. cerevisiae* 20S Proteasome α subunits.

of the seven β-type subunits were found to be synthesised as proproteins, from which the propeptides were cleaved as the proteasome matured. The role that these prosequences play seems to differ between archaebacteria and eukaryotes as the prosequences are not required in archaebacteria for proteasome assembly. In yeast the Doa3 β subunit propeptide was seen to be required for Doa3 incorporation into the proteasome (Chen and Hochstrasser, 1996). The propeptide has been suggested to have a chaperone-like function. This sequence was observed not to be required for proteasome assembly but was an absolute requirement for maturation of specific subsets of active sites. The 20S proteasome is a threonine protease and its active sites lie within the central core of the 20S complex. Processing of the β subunits generates the N-terminal active sites which can cleave peptides following hydrophobic residues (chymotrypsin-like activity), basic residues (tryspin-like activity), and acidic residues (peptidylglutamyl peptide hydrolysing [PGPH]

activity). Three of the β subunits contain active site threonines in comparison to a single active site in *T. acidophilum*. Genetic studies in yeast have suggested that co-operation is required between the β subunits to generate the active sites. These have shown that mutations are required in two subunits for loss of one particular cleavage activity (Kopp *et al.*, 1997). For example in yeast Pre1 and Pre2 are both required for chymotyrpsin-like activity, Pup1 and Pup3 for trypsin-like activity (Arendt and Hochstrasser, 1997), and Pre3 and Pre4 for PGPH activity. Each of the active β subunits contains a hydrophobic pocket that is thought to be the substrate binding pocket as it binds peptide aldehyde inhibitors of the proteasome.

Protein	Size (kDa)	Function
Pre1	23	Degradation of ubiquitinated proteins. Required for cleavage after hydrophobic residues
Pre2/Doa3/Prg1	23*	Functional overlap with Pre1. Has an N-terminal active threonine site
Pre3	21*	Essential for viability and degradation of ubiquitinated proteins. Predicted to have an N-terminal active threonine residue.
Pre4	26**	Functional overlap with Pre3
Pre7	25**	Unknown
Pup1	25**	Predicted to have an N-terminal active threonine residue.
Pup3	23	Unknown

Table 1.6 *S. cerevisiae* 20S Proteasome β subunits. Additional three subunits (LMP2, LMP7, and MECL1) present in human proteasome are not listed. * represents the size of the processed β subunit, and ** represents the size of protein after predicted processing.

The 20S proteasome is assembled as immature precursor complexes of 13S or 16S. The α subunits and unprocessed β subunits are present in the 13S complexes and these can be processed to directly generate the 20S complex. The 16S complex represents the predominant proteasome precursor population and is again composed of α subunits with unprocessed β subunits. A chaperone protein, hsc73 has been found associated with the 16S complex and is thought to function in keeping the complex in a functional conformation (Schmidtke *et al.*, 1997). There is some evidence to suggest that the α subunits form the framework for the correct assembly of the β subunits. The α subunits may also affect the proteolytic activities of the complex. A yeast mutant of the α subunit Pre10 has been observed to have an effect on cleavage ability (Emori *et al.*, 1991). The generation of a precursor complex is thought to prevent the formation of proteolytically active sites until the central hydrophobic chamber has been sealed from the rest of the cell.

The resolution of *S. cerevisiae* 20S proteasome crystal structure at 2.4Å has revealed the structure of this complex. In *T. acidoplilum* two sites of entry into the central proteolytic channel can be found. These are approximately 13Å in diameter and thought to be of a diameter that an unfolded protein could pass through. In contrast to this the proteolytic chamber of the yeast 20S proteasome has been observed to be inaccessible. Groll and colleagues (1997) observed that the N-termini of 5 α subunits project into this opening filling it completely in several layers. No access is available to the interior of this complex without subunit rearrangement. There are smaller side windows present at the interface between the α and β subunits which may be permeable to unfolded polypeptide chains. These openings are approximately 10Å in size and are surrounded by charged residue side chains. This observation has revealed a distinct structural difference between the *T. acidophilum* proteasome and the eukaryotic proteasome and has led to an alteration in the assumed method of peptide entry into the chamber. The studies were performed on purified 20S proteasomes and the effects that the regulatory or activatory complexes exert can only be guessed. It seems reasonable to assume that

as these complexes promote acceleration in peptide degradation and antigen processing that they may function to open up the entry pore of the proteasome *in vivo*.

1.6.4.2 The 19S Cap or Regulatory Complex

In vivo the 20S proteasome has been observed to function in completely breaking down proteins in an ATP-independent manner if the protein is first denatured. This complex is unable to degrade stably folded proteins and requires the addition of an ATP-dependent regulatory complex (RC) to attain this function. Given this observation it seems logical to assume that the function of the RC is to act in unfolding proteins. The RC is positioned in *trans* on either end of the 20S proteasome and generates the 26S proteasome. It is also a multiprotein complex which is composed of at least 15 subunits (Table 1.7). Functionally this complex has been compared to the *E. coli* proteases, ClpAP and ClpXP. ClpA and ClpX are ATP-dependent activators of the ClpP protease. These proteins form a complex that displays some similarity to the 26S proteasome e.g. a central catalytic protease ClpP surrounded on either end by an ATP-dependent activator ClpA or ClpX. In the absence of ClpP, ClpA and ClpX function as chaperones to disassemble multisubunit proteins. These chaperones are thought to act in such a way as to alter protein activity, or if coupled to a protease, destroy the protein (reviewed in Gottesman, 1989).

Six of the components of this complex are members of a family of related ATPases and this accounts for the ATPase activity observed with the RC. These proteins are members of the AAA family (ATPases Associated with a variety of cellular Activities) which are characterised by a highly conserved module of 230 amino acids present in one or two copies in each protein. The proteins within this

family show extensive sequence conservation despite the fact that they display a very diverse range of cellular functions. These range from cell cycle expression, gene expression in yeast and the human immunodeficiency virus (HIV), vesicle mediated transport, peroxisome assembly, and 26S proteasome function (Confalonieri and Duguet, 1995). The six proteasomal ATPases all contain a single conserved domain or ATPase module. Structurally this functional domain is highly conserved e.g. approximately 65% identical between these proteins. Two potential nucleotide binding sites are found within this region. The order of nucleotide preference of the proteasome is ATP>CTP>UTP>GTP (Hoffman and Rechsteiner, 1996b). It has been established that all four nucleotides can support conjugate degradation although only ATP and CTP can promote assembly of the complex. The C-terminal region of the ATPases are also highly conserved in contrast to the N-terminal which is more divergent in sequence. This region of the protein is thought to confer the substrate specificity on the protein (Dubiel *et al.*, 1992). The ATPases within the proteasome are thought to function in unfolding the substrate in readiness for journey through the catalytic core of the complex. They also provide the energy to drive the degradation process although this function could be achieved by a single ATPase. At present it is unclear why so many ATPases are associated with the proteasome. They may function in providing alternative binding sites for particular substrates or they may have functions additional to protein denaturation which have yet to be discovered. It is assumed that a single 26S proteasome complex contains equal amounts of each ATPase, but work from *Manduca sexta* has described an upregulation of a particular subset of ATPases during programmed cell death (Dawson *et al.*, 1994). These workers suggested that multiple populations of 26S proteasomes exist within the cell each containing different ATPase subunits. To date there is little evidence to support this hypothesis and the major assumption from biochemical purification studies is that each RC contains all six ATPases.

Protein	Size (kDa)	Function
Sug1/Cim3	45	ATPase subunit. Required for G2/M cell cycle progression and for the degradation of ubiquitinated proteins.
MSS1/Cim5	52	ATPase subunit. Required for G2/M cell cycle progression.
Sug2/Crl13	49	ATPase subunit. May play a transcriptional role
TBP1/Yta1	48	ATPase subunit. May play a transcriptional role
TBP7/Yta2	48	ATPase subunit. May play a transcriptional role with TBP1.
S4/Yta5	49	ATPase subunit. Required for G2/M cell cycle progression and for the degradation of ubiquitinated proteins.
Nin1	32	Essential for viability and degradation of ubiquitinated proteins. Important for nuclear integrity.
Sen3	104	Essential for viability and degradation of ubiquitinated proteins.
Nas1/Hrd2	109	Low level of similarity to Sen3.
Sun1	30	Ubiquitin chain binding (S5a) subunit. Deletion mutant is viable.
Sun2	60	Essential for viability
Nas2	25	Non-essential subunit.
Nas3	38	Similar to mouse Mov-34 gene product.

Table 1.7 *S. cerevisiae* Regulatory Complex subunits. At least one component of the mammalian 26S proteasome (S5b) is not encoded in the yeast genome.

Of the non-ATPase subunits identified S5a (or SUN-1 in yeast) is the best studied. This protein has been identified as the component of the complex that binds multiubiquitinated proteins. S5a binds only to multiubiquitinated chains and not to free ubiquitin. A highly conserved element within this subunit is thought to represent the sequence that binds ubiquitin chains (Ferrell *et al.*, 1996). Little information on the function of the remaining non-ATPase subunits of the RC is available. S1 or SEN3 in yeast, has been identified as a gene which encodes a factor affecting the tRNA splicing endonuclease system (Dubiel *et al.*, 1995). This would suggest that the 26S proteasome may function in some manner in RNA metabolism. Nin-1 in yeast has been identified as S14 and is shown to be essential for cell viability (Kominami *et al.*, 1995). Mutation of Nin-1 results in an accumulation of ubiquitinated proteins and cells arrested at G1/S and G2/M transition stages. This work suggested that Nin-1 interacted with the cdk CDC28 in S. cerevisiae. The precise function of the remaining proteins of this complex are unknown at present but it seems highly probable that they serve to recognise substrates within this complex.

1.6.4.3 The 11S Activator Complex

Not all 20S proteasomes are assembled into a 26S proteasome complex, some assemble with an 11S activator complex, or PA28. Evidence suggests that both of these proteasome complexes coexist within cells. Each of these complexes serves specific functions: the 26S complexes perform the housekeeping functions e.g. ubiquitin dependent protein degradation. The 20S or 20S-11S proteasomal complexes have been shown to cleave low molecular mass peptidic substrates such as degradation intermediates. The 20S and 20S-11S complexes have been shown to be inefficient in degrading whole proteins and also ubiquitinated proteins (Rechsteiner *et al.*, 1993). The 11S activator is an 180kDa complex which is mainly composed of two homologous subunits, α and β. More recently a third 11S

subunits has been identified. A third protein, γ or Ki antigen, has been identified as homologous to the α and β subunits. All three of these proteins show a high degree of sequence similarity apart from a highly variable internal segment of 17-34 amino acids which may function to confer subunit specific properties (Ahn *et al.*, 1995). The α and β subunits preferentially form a hexameric complex with alternating subunits. Purified α subunits can form heptamers in solution which have biochemical properties similar to the α/β complex but it is unclear whether this occurs *in vivo*. The γ subunit also generates a hexameric ring structure which can associate with the 20S proteasome *in vitro*. At present it is unclear why two types of 11S complexes are present in cells and what the functions of these individual complexes are.

To date the α/β complex is the most widely understood of the 11S activator complexes. Song and co-workers (1997) have demonstrated that one particular residue on the α subunit is required for binding to and activation of the proteasome. Mutations in the carboxyl-terminal tyrosine residue abolished any activity the α subunit displayed previously. α subunit binding to the proteasome and activation of this complex have been shown to be separate events. Analysis of a number of mutant α proteins have identified a loop and a neighbouring proline residue on one face of the α subunit to be critical for proteasome activation (Zhang *et al.*, 1998). In humans the proteasome α subunit C2 contains an epitope which binds to the 11S activator complex. Blocking antibodies have been observed to only partially block 11S activation therefore it seems likely that additional sites are important for activator binding and activity (Kania *et al.*, 1996). β subunits alone have been observed to display no stimulatory effect on proteasome activity but this complex did function in reducing the concentration of α subunit required for activation. These studies suggested that a functional interaction existed between these two 11S subunits. KEKE motifs were identified in the α sequence suggesting a role in interaction with other proteins. Deletion of this motif does not affect subunit

binding to the 20S proteasome suggesting that the KEKE motifs within the 11S activator α subunits do not directly interact with the KEKE motifs observed in some 20S proteasome α-type subunits. It has been hypothesised that these sequence motifs may play a role in the association between α and β subunits (Song *et al.*, 1997). β subunits alone have been observed to display no stimulatory effect on proteasome activity but this complex could function in reducing the concentration of α subunit required for activation. The mechanism by which the β subunits influence α subunit function is unclear, but may be related to protein interactions. The crystal structure of the α/β 11S activator has been examined and this revealed that this complex, much like the 19S RC, may function to cause conformational changes in the 20S proteasome. These changes may facilitate the opening of a channel in the proteasome α subunits through which substrates can pass (Knowlton *et al.*, 1997).

The γ subunit was originally identified as the nuclear protein Ki antigen. Sequence analysis shows this subunit to contain a nuclear localisation signal but studies in HeLa cells have shown that like the α and β subunits its expression is not restricted to one cellular compartment (Tanahashi *et al.*, 1997). The exact cellular function of 11S complexes composed of γ subunits is presently unknown. The expression of α and β subunits is upregulated by IFN-γ. This contrasts to the γ protein which is downreguated by IFN-γ signalling. These initial results suggested that the 11S activator may serve an immunological function. Further evidence for this comes from studies where the α protein was overexpressed. An enhanced presentation of antigenic peptides on MHC class I was observed (Groettrup *et al.*, 1996). The 11S activator complex has been shown to stimulate proteasome peptidase activity but not its ability to stimulate breakdown of full-size proteins. This data has been assumed to suggest that antigen processing by the proteasome may occur in successive steps conducted by various proteasomal complexes (Yang *et al.*, 1995). For example the 26S proteasomal complexes may degrade the ubiquitinated substrate into degradation intermediates which the 20S-11S

proteasome complex further processes into peptides of 8-10 amino acid residues suitable for assembly on MHC Class I proteins. The size of peptide generated by the proteasome is thought to represent the distance between two active sites within the catalytic core of the proteasome. This two step pathway hypothesis matches the observation that only a small number of peptides presented on MHC Class I are degraded by the 26S proteasome. This pathway may be responsible for the generation of peptides from ubiquitinated antigens. The precise genetic dissection of these immuno-proteasome pathways will give more insight into the exact mechansim by which these two complexes interact to generate an immune response to intracellular antigens.

1.6.5 Proteasome Localisation

The subcellular localisation of the proteasome has been examined in a wide range of tissues and a pivotal theme has emerged. In zygotes and early embryos the proteasome is found to be largely cytoplasmic in expression although it becomes increasingly nuclear as development progresses. The nuclear or cytoplasmic distribution of the complex has been observed to vary dependent on the specific tissue examined but a common thought appears to be a more concentrated nuclear expression in dividing or cancerous cells. In non-dividing cells e.g. *Drosophila* salivary glands the proteasome has failed to be detected in the nucleus (Hoffman and Rechsteiner, 1996). These generalisations hold true for the majority of model systems studied. Other components of the ubiquitin mediated degradation system have been found localised in the nucleus. The ubiquitin activating enzyme, E1 is largely nuclear in expression suggesting that a large proportion of intracellular degradation occurs with the nuclear compartment. In addition to this the role that the proteasome plays in the degradation of transcription factors leads to the hypothesis that a particular protein may be stable in the cytoplasm but could be

Tissue/Cell Type	Proteasome Levels	
	μg per milligram cell protein	Molecules per cell
Human lymphocytes	0.135	1.2×10^4
Molt-10 cells	1.36	5.8×10^5
HL60 cells	3.94	1.7×10^6
Human renal cells	7.0	2.9×10^6
Rat muscle	3.27	–
Rat testis	23.6	–
Rat thymus	28.5	1.2×10^7
Rat liver	9.4	4.0×10^6
Rat kidney	2.0	8.5×10^5
Sea urchin egg	–	2.0×10^8

Table 1.8 Levels of the 20S proteasome in Tissues or Cell Types. For lymphocytes it was assumed that 1mg protein = 10^7 cells, and for all other cell types it was assumed that 1mg protein = 2×10^6 cells. (Adapted from Hoffman and Rechsteiner, 1996).

rapidly degraded after entry into the nucleus. If such a compartment specific degradation mechanism exists it provides a highly effective control mechanism for protein stability.

The expression levels of the 26S proteasome are highly varied between tissues. In rat thymus and testes it accounts for 2% of total protein whereas in human lymphocytes it represents as little as 0.01% of proteins (Kumatori *et al.*, 1990). Table 1.8 summarises the expression levels of the proteasome in a range of tissues. The 26S proteasome has been observed to be a relatively stable enzyme. Hendil (1988) demonstrated that 26S proteasomes in HeLa cells had a half-life of approximately 5 days. This constrasts to rat liver where a half-life of 12-15 days was observed (Tanaka and Ichihara, 1989). Expression studies have shown that the proteasome accumulates in cells undergoing developmental processes. These studies have revealed the proteasome to be a highly expressed, highly stable degradation complex.

1.7 Natural Substrates of the Ubiquitin-Proteasome Degradation Pathway

1.7.1 The transcription factor NFκB

The NFκB/Rel family of transcription factors, in response to a wide range of extracellular signals regulate expression of immunoglobulin genes and also participate in T cell activation, HIV infection, and differentiation. NFκB is a heterodimeric transcription factor usually composed of p50 and p65 subunits. In its

inactive form it is sequestered in the cytoplasm and following activation the complex translocates to the nucleus to stimulate gene transcription. NFκB is rendered inactive in two manners. First the p50 subunit exists as an inactive p105 precursor protein. This preprotein is proteolytically processed by the proteasome e.g. the C-terminal prodomain is cleaved. The exact manner in which the proteasome achieves proteolytic processing as opposed to complete degradation is unclear. It has been suggested that the undegraded portion of p105 may fail to enter the proteasome due to a bound ubiquitin group that fails to be cleaved or because this domain of p105 is inherently resistant to unfolding by the ATPases in the 19S regulatory complex. The second mechanism by which NFκB is rendered inactive is by its tethering in the cytoplasm by an inhibitor protein, IκBα. IκBα in response to signalling events can be phosphorylated on two specific serine residues (Ser 32 and 36). This phosphorylation stimulates the ubiquitination of nearby lysine residues and subsequent degradation of the inhibitor. Mutation of either of these serine residues prevents ubiquitination of this protein (Chen *et al.*, 1995). A protein kinase with specificity towards the N-terminal serine residues has been identified (Chen *et al.*, 1996). Phosphorylation of IκBα has been observed to be dependent on ubiquitination of this kinase suggesting that ubiquitination is not only required for protein degradation but also for phosphorylation. The N-termini of p105 and IκBα have been shown to be related in sequence and function by masking the nuclear localisation signal on the p65 subunit of NFκB. In modifying this transcription factor the 26S proteasome has been shown to function in both complete degradation and limited proteolysis.

1.7.2 Cyclin Degradation

Progression of the cell cycle is entirely dependent on the sequential

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1.7.2 Cyclin Degradation

Progression of the cell cycle is entirely dependent on the sequential

activation and inactivation of cyclin dependent kinases (cdks). There are two main classes of cyclins: the mitotic and G1 cyclins. Cyclin B is synthesised during interphase and then rapidly degraded at the end of metaphase. Degradation of this protein allows the cells to exit mitosis and divide. Analysis of cyclin B revealed 8 amino acid residues called the destruction box, which are essential for degradation. Mutation of one residue in the destruction box e.g. Arginine to Cysteine results in stabilisation of cyclin B (Glotzer *et al.*, 1991). The destruction box does not serve as the ubiquitination site of this protein but it may be involved in directing recognition by an E3 enzyme. Cyclin B is associated with cdc2 to form the complete M-phase promoting factor (MPF). A complex of cdc16, cdc23 and cdc27 (called the anaphase-promoting complex), that has been shown to function as an E3 enzyme, interacts with Ubc4 to mediate degradation of the B-type cyclins.

1.7.3 The Tumour Suppressor Protein p53

It has been known for several years that p53 associates with E6 proteins from two strains of human papilloma virus (HPV). The interaction between these two proteins has been observed to result in the ubiquitination and degradation of p53. The p53-E6 complex recruits an additional protein named E6-AP, which acts as an E3 protein ligase, which directly ubiquitinates p53 and the protein is subsequently degraded (Scheffner *et al.*, 1990). The targeting of p53 for this pathway is thought to occur by an N-terminal PEST sequence in addition to protein phosphorylation. This mechanism explains the low levels of p53 protein found in HPV-transformed cervical carcinoma cell lines and contributes to the tumourogenicity of these oncoproteins. p53 is extremely unstable *in vivo* in a range of cell lines which are not infected with HPV suggesting that a normal counterpart of E6 exists. Recently the oncoprotein mdm2 has been identified as binding and catalysing the degradation of p53 *in vivo* (Kubbutat *et al.*, 1997)

1.7.4 MHC Class I Antigen Presentation

MHC Class I molecules carry peptide fragments derived from endogenously expressed proteins from the endoplasmic reticulum to the surface of cells for display to cytotoxic T lymphocytes. Before peptide presentation can occur the cytoplasmic antigens must be cleaved into peptide fragments and transported into the lumen of the endoplasmic reticulum. The system which catalyses this process is the ubiquitin-proteasome pathway. Treatment of cells with IFN-γ results in an increase in expression of three components of the 20S proteasome (LMP2, LMP7, and MECL-1) and also a stimulation of 11S activator complexes. This alteration in proteasome structure results in an enhanced generation of peptides terminating with basic or hydrophobic residues (reviewed in Weissman, 1997). These are the types of peptides preferentially loaded onto MHC Class I molecules. Prior to the discovery of 11S activator complexes it was assumed that the 26S proteasome catalysed the degradation of peptides for this pathway. It is becoming clear that the generation of antigenic peptides by the proteasome is not as simple as assumed. The current hypothesis sugested that only some antigens are fully processed by the 26S proteasome whereas in other situations degradation intermediates are generated. These intermediate degradation products may then be processed to 8-10 amino acid peptides by the 20S-11S proteasome complexes. Mutants in components of the 11S activator complex are required to detail the precise role this complex plays in antigen presentation.

1.8 Protein Degradation by Ubiquitin-Proteasome-like Pathways

1.8.1 Ubiquitin Dependent Endocytosis of Cell Surface Proteins

As a general rule cell surface proteins are rapidly endocytosed and if not recycled to the plasma membrane, are directed to the lysosome where they are degraded by vacuolar proteases (Robinson *et al.*, 1996). This pathway is utilised by the yeast α-factor pheromone receptor, Ste2. Internalised Ste2 was seen to follow an endocytic pathway to the vacuole. Yeast mutants which are defective in receptor endocytosis led to the identification of ubiquitinated forms of Ste2 (Hicke and Riezman, 1996). In support of this, internalisation of ligand bound receptors is inhibited in yeast cells deficient in specific E2 enzymes. Phosphorylation of Ste2 on specific serine residues occurs prior to ubiquitination. A similar pathway has been described for the Ste6 protein suggesting that ubiquitin may be used as a signal for endocytosis and vacuolar targeting. The exact mechanism by which this is achieved is unclear as is the reason why ubiquitinated Ste2 is not targeted to the 26S proteasome. These questions clearly need to be addressed to determine the importance that ubiquitin plays in protein degradation.

1.8.2 Proteasome Dependent Degradation of Endoplasmic Reticulum Associated Proteins

Secretion of proteins is mediated by their uptake into the endoplasmic reticulum (ER). Proteins entering the ER enter in an unfolded state and are subsequently folded and secreted. Inefficiently folded proteins were previously

thought to be eliminated directly in the ER but little was known about the proteolytic system that regulated this process. Recent studies have described a role for the 26S proteasome in the degradation of these ER associated proteins. The first indication of this role of the proteasome came from the study of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an intergral membrane protein which functions by mediating chloride transport across the apical plasma membrane of epithelial cells. Addition of a specific proteasome inhibitor was seen to stimulate production of ubiquitinated CFTR derivatives (Ward *et al.*, 1995). MHC Class I, which is a membrane protein, associates with β2 microglobulin and antigenic peptide in the ER and then transits through the secretory pathway to the plasma membrane for presentation of antigen to cytotoxic T cells. Wiertz and coworkers (1996) demonstrated that a gene product of the human cytomegalovirus (CMV) US11 could target MHC Class I to the cytosol where it was degraded by the proteasome. Again the mechanism by which these two pathways come together is unknown. It is possible that the CFTR is targeted to the proteasome degradation pathway by virtue of its large cytoplasmic domain. The sequence of events thought to occur when these two pathways overlap are glycosylation of the protein in the ER followed by retrograde transport out of the ER, possibly in association with a chaperone protein. The protein is then tagged by ubiquitin and degraded as normal by the proteasome.

1.8.3 Ubiquitin-Free Proteolysis by the 26S Proteasome

As a general rule all proteins degraded by the 26S proteasome were thought to require ubiquitination prior to their binding to the regulatory complex. Studies of the metabolic enzyme ornithine decarboxylase (ODC) have disputed this claim. ODC is involved in polyamine synthesis and is degraded in an ATP-dependent manner by the proteasome. The prime difference between this protein and others

which are degraded by the proteasome is that it is not ubiquitinated prior to breakdown. In constrast ODC targeting to the proteasome is dependent on its association with another protein, antizyme. This interaction results in the destruction of ODC, while sparing antizyme. How this degradation is catalysed without the presence of ubiquitin is unknown but it clearly suggests that this degradation pathway contains many more control mechansims than have been identified at present.

1.8.4 Ubiquitin-Like Modifying Proteins

A number of ubiquitin related proteins have been discovered in yeast as well as other organisms in recent years. Although the sequence of these ubiquitin-like proteins has highly diverged from ubiquitin they were named as such due to their ability to be posttranslationally conjugated to other proteins. The best defined role for ubiquitin lies in facilitating degradation of proteins by the 26S proteasome but it has also been shown to be crucial in altering protein function in mechansims distinct from the proteasome. For example histone H2A when ubiquitinated is a relatively stable protein. Ubiquitin tagging is also found to be essential for receptor internalisation and transport to the lysosome (described in section 1.8.1) as well as for activating a protein kinase which phosphorylates IκBα *in vitro* (described in section 1.7.1).

The best studies of the ubiquitin-like (Ubl) proteins is the mammalian ubiquitin cross-reactive protein (UCRP). UCRP exists as a preprotein containing two domains which are related to ubiquitin. Ligation of UCRP to its substrate, which has yet to be identified, results in localisation to intermediate filaments. The

ability of this protein to form thiolester bonds is extremely inefficient suggesting that a specific UCRP activating enzyme may exist (Narasimhan *et al.*, 1996).

The other major Ubl studied to date is SUMO-1 (small ubiquitin-related modifier). SUMO-1 (SMT3 in *S. cerevisiae*) is a 101 residue protein which is 18% identical to ubiquitin. Sequence comparisons of EST (expressed sequence tag) databases have suggested that there are at least two addition variants of SUMO-1, named SUMO-2 and SUMO-3. Like UCRP, SUMO-1 is ligated to other proteins and some of these substrates have been identified. It associates with the PML proto-oncogene, the human DNA repair proteins Rad51 and Rad52, and the cell death domain of Fas (reviewed in Saitoh *et al.*, 1997a). SUMO-1 was also discovered as a modifier of the GTPase-activating protein of Ran, RanGAP1 and the function of SUMO-1 has been analysed in more detail within this pathway. Ran is a small GTPase required for nuclear transport. To facilitate an enhanced rate of GTP hydrolysis an activating protein, RanGAP-1 is required. Two forms of RanGAP-1 are found in vertebrates: an unmodified 70kDa form and a 90kDa form conjugated to SUMO-1. Conjugation of SUMO-1 to RanGAP-1 is required to target RanGAP-1 to its binding protein, RanBP-2. This modification of these proteins is essential for import of the complex through nuclear pore complexes (Mahajan *et al.*, 1997). The precise mechanism by which SUMO-1 modifies RanGAP-1 to allow targeting to the nuclear pore is unknown. Two hybrid analysis has revealed that SUMO-1 associates with the death domains of Fas/APO-1 and tumour necrosis factor (TNF) receptors, all of which are involved in signalling apoptosis. Mutations inactivating the death domains of these proteins prevent an interaction with SUMO-1 and inhibit apoptosis (Okura *et al.*, 1996).

In a manner similar to ubiquitin SUMO-1 also requires processing to cleave four amino acids from its C-terminus prior to substrate conjugation. The enzymes which are involved in SUMO-1 conjugation to substrate are not fully characterised.

The strongest candidate for an E2 which acts on SUMO-1 is the Ubc9 enzyme. In *Xenopus* Ubc9 forms a complex with SUMO-1, RanGAP-1 and RanBP-2 (Saitoh *et al.*, 1997b). Desterro and colleagues (1997) have shown that human Ubc9 could form a thioester with SUMO-1 but not with ubiquitin suggesting that Ubc9 is a SUMO-1 conjugating enzyme rather than a ubiquitin conjugating enzyme. These observations would suggest that Ubl proteins may be conjugated to substrate by different enzymes which function in a similar pathway to ubiquitination. The common theme between ubiquitin and ubiquitin-like proteins is that they both function by modifying a protein. This modification targets the substrate to a specific structure or site within the cell. Many fundamental questions pertaining to the function of this system remain unanswered, and these should be addressed when more substrates of Ubl conjugation are discovered.

1.9 Proteolysis Overview

Protein turnover is an essential process in controlling the regulation of the cellular environment. It is necessary for a cell to undergo changes in response to specific stimuli, to remove damaged or incorrectly folded proteins from the cell, and to allow a cell to adapt to developmental changes. The 26S proteasome is the prime mechanism within the cell for the complete breakdown of short-lived intracellular proteins. It is a complex pathway composed of many regulatory steps, some of which have been described above. In the last decade a plethora of information has become available on the proteins involved in this degradation pathway and this has revealed a detailed picture of the system. Due to the high evolutionary conservation of the proteasome much of this information has been elucidated from studies on yeast mutants of these proteins. Whilst providing a basis for the understanding of this degradation pathway these studies also reveal whether mutation of one component of this pathway can disrupt protein degradation. One aspect which yeast

analysis cannot address is whether the proteasome has a role in developmental processes. Genetic dissection of this pathway in a number of developing systems, mainly *Drosophila*, have implied that the 26S proteasome may play a prime role in regulating development. At present little is known regarding how this system functions to regulate development in a mammalian system. It seems highly likely that the next stage in proteasome research will be the description of the genetic dissection of the mouse proteasome pathway. This will almost definitely reveal a new set of functions for the 26S proteasome which had not previously been considered.

1.10 Aims of the Project

The aims of the studies reported in this thesis were to analyse gene expression during development of the mammary gland. It was hoped that by using this approach we would identify genes that were involved in controlling the differentiation process within the gland. The outcome of this study was the identification of a gene that was indeed upregulated during development of the mammary gland. The gene identified was a component of a protein degradation pathway, which had not previously been considered to play a role in mammary gland development. We have shown that this degradation pathway is essential for normal functioning of mammary epithelial cells and thus have achieved an aim set out at the onset of this project.

Chapter 2

Materials and Methods

Unless stated otherwise reagents were obtained from Sigma (Poole, United Kingdom).

2.1 DNA Manipulation and Analysis

2.1.1 Restriction Digest of Plasmid DNA

Plasmid DNA was typically digested at 37°C for 2 hours using the manufacturers suggested restriction buffer (Boehringer Mannheim, New England Biolabs [NEB]) with 3 times excess of the recommended amount of enzyme. Double digests were carried out simultaneously provided the salt concentrations in the buffers were compatible, otherwise the digest at the lower salt concentration was carried out first and the salt concentration subsequently adjusted to meet the conditions for the second enzyme digest. PCR products generated containing 5' and 3' restriction sites were routinely digested overnight with the first enzyme, then subsequently with the second enzyme as this PCR generated DNA generally digested less efficiently due to shorter flanking sequence at one end of the restriction site.

2.1.2 Purification of DNA Fragments from Agarose Gels

Plasmid DNA fragments were resolved on low melting point (LMP) agarose gels and the fragment of interest excised from the gel and recovered using β Agarase (NEB) as described by the manufacturers. 10μg DNA was digested and electrophoresed in LMP agarose. The DNA was visualised using long-wave UV light and the appropriate fragment excised from the gel. This gel fragment was heated to 65°C in 1/10 volume of buffer until the agarose was molten and then transferred to 40°C for 1 hour with 2 units of β agarase. Following agarose digestion the DNA was concentrated by the addition of 1/10 volume of 3M sodium acetate (NaAc) and 3 volumes of isopropanol. DNA was pelleted by centrifugation and

washed with 70% ethanol to remove salt, air dried and resuspended in ddH_2O. Approximately 50% of the original digested DNA was recovered using this method.

2.1.3 Creation of Blunt Ended DNA

This procedure is dependent on the exonuclease activity of DNA Polymerase I. The C-terminal Klenow fragment can be used to fill recessed 3' termini created by restriction digestion of plasmid DNA. Conversely, it can also be used to fill recessed 5' termini. This latter reaction occurs in two stages. Initially the 3'→5' exonuclease activity removes protruding 3' tails from the DNA. This creates a recessed 3' terminus which can be blunt ended by the incorporation of nucleotides at this 3' terminus. Thus this enzyme can be used to generate blunt ended DNA regardless of where there is a 3' or 5' recessed termini. The reaction was as follows:-

500ng Insert DNA
1.5µl 10 x Klenow Buffer
1.5µl 133µM dNTPs
0.5µl Klenow (2U/µl)
to 15µl total volume

The reaction was incubated at 37°C for 1 hour and then stopped by heat inactivating the enzyme at 75°C for 20 minutes. The DNA was concentrated by precipitation with 1/10 volume of 3M NaAc and 3 volumes of ethanol. Following centrifugation of the DNA, the pellet was washed in 70% ethanol, air dried and resuspended in 20µl ddH_2O.

2.1.4 Ligation Reactions

For cohesive end ligations, approximately 50-100ng vector and insert DNA were used in a molar ratio of 1:3 in a 10µl reaction containing T4 DNA Ligase (Boehringer Mannheim) and reaction buffer. This reaction was incubated at 16°C

overnight and 2µl used to transform *E.coli* DH5α competent cells. For blunt end ligations 300ng DNA was used and the reaction was incubated at 4°C.

2.1.5 Transformation of Competent Cells with Plasmid DNA

E.coli DH5α competent cells (Genotype: F^-, endA1, hsdR17[rk^-,mk^+], supE44, thi-1, λ-, recA1, gyrA96, relA1,Δ[argF-lacZYA]U169, ϕ80dlacZΔ[M15Δ]) {purchased from Life Technologies Inc} were defrosted on ice and 20µl of cells were transformed with 2µl of the ligation reaction according to manufacturers instructions. The transformation reaction was plated onto LB plates (1% w/v yeast extract, 1.5% w/v agar, 0.1M NaCl) containing ampicillin at a concentration of 50µg/ml and Xgal and IPTG (40µg/ml and 60µg/ml respectively). Transformants were selected by blue/white colour selection for the Bluescript and PCRII cloning Vectors. These vectors contain a sequence, which encodes the α fragment of the lacZ gene into which a polylinker is inserted. The colour selection exploits the α-complementation process to select recombinant plasmids. Non-recombinant plasmids produce a functional α fragment (N terminus) and when the host cells produce a ω fragment (C terminus) through the lacZ[M15Δ] marker, a functional β-galactosidase is produced. When cells are plated on media containing IPTG, which inactivates the lacZ repressor, and Xgal, the substrate for β-galactosidase, the colonies are blue. If there is DNA inserted into the polylinker then this disrupts the α fragment and α-complementation cannot occur, therefore the recombinant colonies are white. For cloning of PCR products into the TA cloning vector (Invitrogen) the above procedure was used to transform Top10F' cells (Genotype F' {$lacI^qTh10(Tet^R)$} mcrAΔ [mrr-hsdRM-mcrBC],ϕ80lacZΔM15Δ, lacX74, recA1, ara, D139Δ(ara-leu)7697, galU, galK, $rpsL(Str^R)$, endA1, nupG).

2.1.6 Small Scale Purification of Plasmid DNA

Plasmid DNA was purified using the alkali lysis method described by Sambrook *et al*, (1989). A 2ml culture containing 50μg/ml ampicillin was inoculated with a single colony and incubated at 37°C overnight in a shaking incubator. 1.5ml of each sample was transferred to an eppendorf tube and centrifuged to pellet the bacterial cells. Following removal of the supernatant, the pellet was resuspended in 100μl of ice cold TGE (25mM Tris-HCl pH8.0, 50mM Glucose, 10mM EDTA pH8.0) and incubated on ice for 10 minutes. 240μl of 0.2M NaOH/1% (w/v) SDS was added to lyse the cells. Samples were inverted to mix the solution and incubated at room temperature for 5 minutes before addition of 120μl of ice cold 5M Potassium Acetate (KAc). Samples were incubated on ice for 10 minutes following inversion to mix. Bacterial cells were centrifuged at 13000rpm for 10 minutes, and the DNA containing supernatant was transferred to a fresh tube where it was precipitated by the addition of 0.6 volumes of isopropanol. The DNA was centrifuged at 13000rpm for 10 minutes and washed in 70% ethanol. The pellet was air dried prior to resuspension in ddH_2O containing DNAse-free pancreatic RNAse A (20μg/ml).

2.1.7 Large Scale Purification of Plasmid DNA

Large scale bacterial cultures were purified by a modified alkali lysis method (Birnboim and Doly, 1979) and followed by caesium chloride gradient centrifugation. A 250ml culture of LB medium containing ampicillin at 50μg/ml was inoculated with 1ml of a pre-culture grown at 37°C for 8 hours, and incubated at 37°C overnight in a shaking incubator. The bacteria were pelleted by centrifugation at 6000rpm at 4°C for 10 minutes in a Beckman JA-20 rotor. The cell pellet was resuspended in 10ml of ice cold TGE buffer and incubated on ice for 10 minutes. Cells were lysed by the addition of 10mls of 0.2M NaOH/1% (w/v) SDS. The solution was incubated at room temperature for 10 minutes and chromosomal DNA

and protein were precipitated by the addition of 7.5mls of 5M KAc. The solution was incubated on ice for 10 minutes followed by centrifugation at 6000rpm in a Beckman JA-14 rotor. The DNA containing supernatant was transferred to a fresh tube through 6 layers of gauze and DNA was precipitated by the addition of 0.6 volumes of isopropanol. Plasmid DNA was pelleted by centrifugation at 9000rpm for 10 minutes and the pellet washed twice in 70% ethanol. The DNA was air dried and resuspended in 2.6mls ddH_2O.

2.1.8 Caesium Chloride/Ethidium Bromide Density Gradient Purification of Plasmid DNA

Purified plasmid DNA (2.6mls) was added to 2.7g Caesium Chloride ($CsCl_2$) and dissolved at room temperature. 220µl of 10mg/ml Ethidium Bromide was added and mixed with the DNA. The solution was then transferred to a Beckman Quickseal tube and centrifuged at 70000rpm for 18 hours at 20°C in a Beckman Ti60.5 rotor. Following centrifugation three distinct bands were visualised. The closed circular and supercoiled DNA, which corresponded to the lower of the three bands, was removed from the tubes using an 18 gauge needle and syringe. The ethidium bromide was extracted from the solution by adding an equal volume of iso-amyl alcohol and vortexing for 1 minute. This was followed by centrifugation at 13000rpm for 5 minutes. The upper phase containing ethidium bromide was removed and the procedure repeated until all ethidium bromide was completely removed, requiring a minimum of 6 iso-amyl alcohol extractions. DNA was diluted in 2 volumes of ddH_2O and precipitated with 3 volumes of ethanol. Centrifugation at 13000rpm for 15 minutes pelleted the DNA, which was then washed in 70% ethanol and resuspended in 250µl ddH_2O. The precipitation procedure was repeated to ensure the DNA was free from any contaminating $CsCl_2$ and the DNA resuspended in a final volume of 400µl. $CsCl_2$ plasmid preparations were used when high quality pure DNA was required, in particular for transfection into cultured cells.

2.1.9 Agarose Gel Electrophoresis of Plasmid DNA

1g (w/v) of agarose was dissolved in 1 x TAE buffer (40mM Tris-acetate, 2.5mM EDTA pH7.7) and allowed to cool to 60°C before addition of Ethidium Bromide at a concentration of 1μg/ml. The gel was allowed to set before submersion in 1 x TAE running buffer. Each sample was diluted in 1/10 volume of loading dye and was electrophoresed at 100V for 2 hours. The gel was visualised under long wave UV illumination and photographed.

2.1.10 DNA Transfer from Agarose Gels

Plasmid DNA from agarose gels was transferred onto Hybond-N nylon membrane (Amersham) using the method described by Southern (1975). DNA was denatured in a solution of 1.5M NaCl, 0.5M NaOH for 15 minutes at room temperature on a rocking platform. This step was then repeated and followed by three washes in neutralising solution (1.5M NaCl, 0.5M Tris pH7.5, 1mM EDTA). The gel was placed on a wick composed of a sheet of Whatman 3mm paper soaked in 10 x SSC (1.5M NaCl, 0.15M sodium citrate) overhanging the buffer reservoir. This was overlaid with a piece of Hybond-N membrane cut to the size of the gel and 10 pieces of 3mm paper, 5 of which were soaked in 2 x SSC. Air bubbles were removed by rolling a glass pipette over the gel, membrane and 3mm paper. Paper towels were then placed on top, followed by a glass plate and a weight. This was left overnight allowing the DNA to transfer to the membrane by capillary action. The membrane was removed and rinsed in 2 x SSC, allowed to air dry and baked at 80°C for 2 hours to fix the DNA to the membrane. If the membrane was not used immediately, it was wrapped in Saran-wrap and stored at 4°C until use.

2.1.11 Double Stranded DNA Sequencing

Using a T7 sequencing kit (Promega), double stranded DNA was sequenced according to the method of Sanger *et al*, (1977). 1.5μg of template DNA in a total volume of 32μl was denatured by the addition of 8μl 2M NaOH and incubated at room temperature for 10 minutes. DNA templates were then diluted with 1/10 volume 3M NaAc and 2 volumes of ethanol. This mix was chilled at -20°C for 15 minutes and precipitated by centrifugation at 13000rpm for 15 minutes. The DNA pellet was washed in 70% ethanol, air dried and resuspended in 10μl dH_2O. The DNA template and 2μl primer (10pmol) were annealed by the addition of 2μl annealing buffer and incubating for 5 minutes at 65°C, followed by a 10 minute incubation at 37°C. The annealed primer and DNA mix were then left at room temperature for a further 5 minutes before commencing the sequencing reactions. 2.5μl of A, G, C, and T short-run termination mixes were aliquoted into eppendorf tubes and pre-warmed to 37°C prior to addition of the labelling reaction. 6μl of labelling reaction was added to the annealed primer/DNA mix and incubated at room temperature for 5 minutes. The labelling mix contained the following components:-

dATP Labelling Mix	3n μl
Diluted T7 DNA Polymerase	2n μl (2U/μl)
α ^{35}S dATP	n μl (Amersham, specific activity 1000Ci/mmol)
Total volume	6μl, where n = number of templates sequenced

4.5μl of labelling mix was added to the pre-warmed termination mixes and incubated at 37°C for 5 minutes. This reaction was terminated by the addition of 5 μl of stop solution.

7M Urea gels containing 6% polyacrylamide in 1 x TBE buffer (0.13M Tris HCl, 4mM Boric acid, 0.25mM EDTA) were routinely used for DNA sequencing. Sequencing reactions were denatured at 80°C for 2 minutes prior to loading on the gel, and electrophoresed at a constant power of 70 watts in 1 x TBE running buffer. Gels were fixed by soaking in 10% methanol (v/v), 10% acetic acid (v/v) for 15

minutes prior to drying at 80°C for 1 hour. The dried gel was then exposed to autoradiographic film overnight (Kodak MR1 Film). Approximately 150-200bp of sequence could be read from a single run.

2.2 RNA Extraction and Analysis

2.2.1 Total RNA Isolation from Mouse Tissue

All plastics used for RNA analysis were double autoclaved and glassware was baked at 180°C prior to use. Dissected organs were ground to a powder in liquid nitrogen and homogenised in RNAzol (Biogenesis) at 2mls per 250mg tissue. 1/10 volume of chloroform was added to the samples, mixed and incubated on ice for 5 minutes. Following centrifugation at 13000rpm for 15 minutes at 4°C, the upper aqueous phase was removed and protein and the inorganic lower layer was discarded. RNA was precipitated by the addition of an equal volume of isopropanol and incubated at 4°C for 15 minutes. This was followed by centrifugation at 13000rpm for 15 minutes at 4°C to pellet the RNA, which was then washed in 70% ethanol, air dried on ice and resuspended in ddH_2O. For long-term use RNA was stored as an ethanol precipitate at -20°C.

2.2.2 RNA Isolation From Cultured Cells

Confluent T80cm^2 flasks of cells (approximately 3 x 10^7 cells) were rinsed in PBS and the monolayer harvested by trypsinisation (section 2.4.1). The cells were pelleted by centrifugation at 1000rpm for 5 minutes and the supernatant removed. On ice the cells were resuspended in 4mls RNAzol and the RNA extracted as above.

mRNA was isolated from cultured cells using an Invitrogen Micro-FastTrack Kit as described by manufacturers. Briefly, 1 x 10^6 cells were resuspended in 1ml lysis buffer and incubated at 45°C for 20 minutes to digest protein and RNAses. The salt concentration of the samples was adjusted to 0.5M with NaCl and DNA was sheared by passing the lysate through an 18 gauge needle three times. One oligo dT cellulose tablet was added to the lysate and allowed to dissolve. Once dissolved the solution was incubated at room temperature for 20 minutes on a rocking platform. The oligo dT cellulose was subsequently pelleted at 13000rpm for 5 minutes, and the resultant pellet washed three times in binding buffer. The oligo dT cellulose was resuspended in 0.3mls of binding buffer and applied to a spin column. The solution was washed three times in binding buffer and the non-polyadenylated RNA was eluted from the column by two washes in low salt buffer. mRNA was eluted in 200µl elution buffer by centrifugation at 13000rpm for 1 minute. mRNA was stored as an ethanol precipitate at –70°C by the addition of 10µl glycogen (2mg/ml), 30µl 2M NaAc, and 600µl 100% ethanol.

2.2.3 Formaldehyde Gel Electrophoresis of RNA

Both total RNA and poly A^+ RNA samples were electrophoresed on a 0.8% (w/v) agarose gel dissolved in 1 x Mops buffer (0.02M 3-N-[morpholinol] propanesulfonic acid, 5mM NaAc, 1mM EDTA pH7.0) containing 6% (v/v) formaldehyde. 10µg of total RNA (or 2µg of mRNA) was centrifuged at 13000rpm for 30minutes at 4°C to pellet the RNA, which was then washed in 70% ethanol. The resultant pellet was dried on ice and resuspended in 30µl RNA sample buffer (15µl Formamide, 5µl 10 x Mops buffer, 8µl 38% formaldehyde, and 2µl ddH_2O). 1/10 volume of loading dye and 0.5µl of 10mg/ml Ethidium Bromide was added to samples prior to denaturing at 65°C for 10 minutes. RNA was then immediately loaded onto the gel, which was submerged in 1 x Mops running buffer and electrophoresed at 65V for 4 hours. RNA was visualised under long wave UV and photographed.

2.2.4 RNA Transfer from Formaldehyde Gels

RNA transfer was similar to that described in section 2.1.10 with the following exceptions. No denaturisation or neutralisation of the gel was necessary. A capillary transfer was performed using Zetaprobe nylon membrane (BioRad) and the transfer buffer was composed of 0.04M NaOH, 0.5M NaCl. Following transfer the membrane was rinsed in 2 x SSC and was baked at 65°C for 2 hours to fix the RNA to the membrane.

2.3 Radiolabelling and Hybridisation Techniques

2.3.1 Random Priming

Radiolabelled DNA probes were prepared by the methods described by Feionberg and Vogelstein (1984). This procedure exploits the lack of 5'→3' exonuclease activity of Klenow, and therefore incorporates nucleotides complementary to 5' DNA overhangs. 50ng of linearised double stranded DNA was diluted to 24µl in ddH_2O and added to 10µl of random nonomeric primers (27OD units/ml) from a Prime-It II Kit (Stratagene). The DNA was then denatured at 95°C for 5 minutes followed by chilling on ice. The following components were then added:-

10µl 5 x dCTP Buffer

1µl Klenow fragment of DNA Polymerase I (5U/ml)

5µl α^{32}P dCTP (Amersham, specific activity 3000Ci/mM)

The reaction was incubated at 37°C for 30 minutes. Unincorporated nucleotide was removed by diluting the probe to 100µl with ddH_2O and applying to a G50 Sephadex column (5'→3' Inc) according to manufacturer's instructions. Briefly, the column was inverted to mix the resin and centrifuged at 1000rpm for 2 minutes to remove

the storage buffer from the column. The radiolabelled DNA was then applied to the column and centrifuged at 1000rpm for 2 minutes. Unincorporated nucleotides remained trapped in the resin and labelled DNA fragments larger than 50bp were eluted from the column. The specific activity of the probe was measured by quantitation of an aliquot using a scintillation counter. The probe was denatured prior to hybridisation by the addition of 1/4 volume 2M NaOH and left at room temperature for 2 minutes before addition to the hybridisation solution.

2.3.2 Hybridisation of DNA Probes to Nylon Membranes

The Church and Gilbert (1984) method was followed for hybridisation. Membranes were prehybridised at 65°C for 30 minutes in 15mls of hybridisation solution (0.5M $NaPO_4$ pH7.0, 7% w/v SDS, 1mM EDTA) in a roller oven. Labelled DNA probe was added at an activity of 1 x 10^6 cpm/ml of hybridisation solution and incubated overnight at 65°C. The excess unhybridised probe was removed using a range of salt washes. Low stringency washes were performed using two incubations in pre-warmed 40mM $NaPO_4$, 1% SDS at 65°C. If non-specific background was high, two further washes in 20mM $NaPO_4$/1% SDS were performed. The membrane was wrapped in Saran-wrap and exposed to X-ray film overnight at -70°C with intensifying screens.

2.3.3 RNAse Protection Assay

A Maxiscript *In Vitro* transcription Kit (Ambion) was used to generated RNA transcripts. 1µg of cloned plasmid DNA was linearised for transcription from T7 and Sp6 polymerase promoters. The reactions were set up as follows:

2µl 10 x Transcription Buffer

1µl 200mM DTT

1µl 10mM ATP

1µl 10mM CTP

1µl 10mM GTP

1µl RNAse Inhibitor

1µg linearised template DNA

3µl 0.1mM UTP

5µl α^{32}P dUTP (Amersham, specific activity 3000Ci/mmol)

1µl SP6 or T7 RNA Polymerase (10U/µl)

ddH_2O to 20µl.

Reactions were incubated at 37°C for 30 minutes. 1µl RNAse-free DNAse I (2U/µl) was added and incubated at 37°C for 15 minutes to remove template DNA. An equal volume of gel loading buffer was added to the samples, which were subsequently denatured at 90°C for 5 minutes. The reaction was loaded onto an 8M Urea/5% acrylamide gel and run for 1 hour at 100 volts. After electrophoresis, the gel was wrapped in Saran Wrap and exposed to film for 2-3 minutes. Following development of the autoradiograph, the full-length transcript was identified as the most intense band present. This film was used as a template to cut the corresponding area from the gel. The gel slice was diluted in 350µl elution buffer (0.5M NH_4Ac, 1mM EDTA, 0.1% [w/v] SDS) and incubated at 37°C overnight. The gel fragment was removed from the sample and the quantity of radioactive label in the aliquot was determined by scintillation counting.

The RNase Protection Assay was performed using an RPA II Kit (Ambion) according to the supplier's instructions. 10µg total RNA was mixed with 6 x 10^4cpm of *in vitro* transcribed probe. Samples were diluted to 50µl and ethanol precipitated by adding 1/10 volume 3M NaAc and 2.5 volumes of ethanol. Following 15 minutes at –20°C, samples were centrifuged at 13000rpm for 15 minutes at 4°C. RNA was washed, air dried and subsequently resuspended in 20µl hybridisation buffer (80% v/v deionised formamide, 40mM PIPES pH 6.4, 400mM NaAc pH 6.4, 1mM EDTA). The RNA and probe suspension was denatured at 99°C for 3minutes prior to hybridisation at 42°C overnight. 200µl of RNase Digestion Buffer (containing diluted RNase A and RNase T1 mixture) was added to the samples and incubated at

37°C for 30 minutes to digest unprotected single-stranded RNA. 300µl of RNAse Inactivation/Precipitation mixture was added to the samples and vortexed to mix. Samples were chilled at –20°C for 15 minutes, prior to centrifugation at 13000rpm for 15 minutes at 4°C. Pellets were resuspended in 8µl of loading dye and denatured at 99°C for 3 minutes. Protected RNA fragments were separated on a 5% acrylamide/8M urea gel in 1 x TBE running buffer at 250 volts for 3 hours. The gel was transferred onto 3mm paper and dried at 80°C for 1 hour, prior to exposure to autoradiographic film at –70°C overnight with intensifying screens.

2.4 Culture of Murine Mammary Epithelial Cells

2.4.1 Passaging and Maintenance of KIM-2 Cells

KIM-2 mammary epithelial cells (MECs) were grown as a monolayer and routinely passaged every 3-4 days. For passaging, media was aspirated from the cells which were then rinsed in sterile PBS (Gibco BRL). 2.5mls of prewarmed TEG solution (per T80cm^2 flask) was added to the cells and left for 1 minute. 10mls maintenance media was added to the cells to inactivate the trypsin, and the cells were harvested using a cell scraper (Costar). KIM-2 cells were routinely passaged as clumps of 5-10 cells, which were generated by a minimal pipetting of the cells once they were in suspension. Cells were collected by centrifugation at 1000rpm for 5 minutes. The supernatant was aspirated and the cells were resuspended in an appropriate volume of maintenance media and dispersed by gentle pipetting. Cells were routinely split 1 in 3 every 3 to 4 days with 1 flask always remaining on collagen. Cells were incubated at 37°C with 5% CO_2. Media was replaced on the cells 24 hours following passaging and thereafter every 2 days.

Maintenance Media

1:1 DMEM/F12 (Gibco BRL)

10% (v/v) FCS

5µg/ml Bovine Pancreatic Insulin

10ng/ml EGF

5µg/ml Linoleic Acid/BSA

6ml L-Glutamine (Gibco BRL)

TEG (Trypsin EGTA)

NaCl	6.3g
Na_2HPO_4	0.12g
KH_2PO_4	0.216g
KCl	0.333g
D-Glucose	0.9g
Tris Base	2.7g
EGTA	0.4g
PVA (Polyvinyl Alcohol)	0.1g
1% (v/v) Phenol Red	0.9ml
2.5% (v/v) Trypsin	100mls (ICN Flow)

This solution was adjusted to pH7.6, diluted to 1 litre, filter sterilised and stored at -20°C.

Collagen Coated Flasks

5mls Type I Collagen from Calf Skin was added to 2.8mls 10% (v/v) acetic acid, and diluted to 50mls in ddH_20. 10mls of this solution was added to a T80cm^2 flask and incubated at 4°C for at least 24 hours. The collagen solution was aspirated from the flask and followed by three washes in PBS. The flask was left to rinse in PBS for 30 minutes at room temperature, followed by a minimum of three washes in maintenance media to remove any residual acetic acid.

2.4.2 Induction of Differentiation in KIM-2 Cells

KIM-2 cells were passaged as described above and grown until reaching confluency, whereupon media was changed and left for a further 48 hours before the cells were induced to differentiate. Differentiation media was maintenance media minus EGF but with the addition of Dexamethasome (1μM, Gibco BRL) and ovine Prolactin (5μg/ml). Cells were treated with prolactin and glucocorticoid for up to a maximum of 12 days. Cells were routinely harvested to check functional differentiation as measured by the expression of the milk protein β casein.

2.4.3 Freezing KIM-2 Cells

Cells were harvested by trypsinisation from a T80cm^2 flask as described in section 2.4.1. The pellet was resuspended in 2mls maintenance media and placed on ice. An equal volume of ice cold freeze mix was added drop-wise to the cells whilst mixing the contents. This mixture was then aliquoted into four 1ml aliquots in cold freezing vials and placed on -70°C overnight. The cells were subsequently transferred to liquid nitrogen for long term storage.

Freeze Mix

60% (v/v) maintenance medium

20% (v/v) FCS

20% (v/v) dimethyl sulfoxide [DMSO]

To defrost cells from liquid nitrogen, the frozen vial was warmed to 37°C until melted. The cells were then diluted in 10mls maintenance media and centrifuged to remove the DMSO. The pellet was resuspended in 5mls media and seeded in a T25cm^2 flask at 37°C with 5% CO_2.

2.4.4 Passaging and Maintenance of HC11 Cells

HC11 mammary epithelial cells were grown as a monolayer and passaged every 3-4 days in a similar manner to the KIM-2 cells (section 2.4.1). The main differences were that HC11 cells did not require to be passaged on collagen, and they could be split as single cells. The maintenance media for HC11 cells was RPMI 1640 (++) and this was supplemented with the same growth factors as KIM-2 media except twice the concentration of EGF was required. HC11 cells were differentiated in a similar manner as above except the serum concentration was reduced to 3% as opposed to 10% for the KIM-2 cells.

2.4.5 Transfection of KIM-2 Cells

KIM-2 cells were routinely transfected using 2μg DNA, which had been purified twice by $CsCl_2$ density gradient centrifugation using a lipid based transfection reagent, Fugene (Boehringer). Cells were transfected 24 hours following passaging for various lengths of time (maximum of 24 hours). Transfected cells were then selected using the appropriate antibiotic, usually geneticin for 14 days is stable clones were to be generated. The resultant clones were expanded until a frozen stock of these cells could be generated. Transfection efficiencies were monitored using a plasmid containing a Lac Z gene. Following transfection these test transfections were fixed in solution 1 at room temperature for 10 minutes prior to overnight incubation in solution 2 at 37°C. The number of blue cells were subsequently counted to determine transfection efficiencies.

Solution 1 (in PBS)	Solution 2 (in PBS)
2mM MgCl2	5mM Potassium Ferracyanide
5mM EGTA	2mM Potassium Ferrocyanide
0.2% Gluteraldehyde	1mg/ml X-gal

2.5 Culture of Murine Embryonic Stem Cells

Murine embryonic stem (ES) cells were cultured in a manner similar to KIM-2 mammary epithelial cells. Cells were approximately 60% confluent when passaged, this prevented the cells initiating differentiation which occurs when they become confluent. Similar to KIM-2 cells, these cells were rinsed in sterile PBS prior to incubation in a trypsin solution for 5 minutes at 37°C. Cells were resuspended in a single cell suspension prior to re-plating in a larger flask. ES cells were grown as a monolayer on gelatinised flasks (0.1% gelatin solution) at 37°C in 5% CO_2 and media was replaced every second day following passaging.

ES Cell Maintenace Media

BHK21 media (Glasgow MEM, Gibco BRL)

10% Serum (1:1 of FCS and Newborn Serum)

6ml L-Glutamine (Gibco BRL)

500µl Recombinant LIF (Leukaemic Inhibitory Factor)

500µl β-2mercaptoethanol (β-2M) solution (1:142 dilution of β-2M in ddH_2O)

2.6 Cell Cycle and Apoptosis Analysis

2.6.1 Cell Cycle Synchronisation

KIM-2 cells were split 1 in 3 in maintenance media and incubated at 37°C for 24 hours. The cells were then treated with the following drugs:-

a) Hydroxyurea (HXU) which blocks cells late in the G1 phase of the cell cycle. HXU was diluted in maintenance media and a concentration of 1mM used to arrest KIM-2 cells.

b) Nocodazole which blocks cells in the G2/M phase of the cell cycle. Nocodazole was diluted in DMSO and 50ng/ml used to arrest KIM-2 cells.
Control cells were treated with the appropriate diluent alone and incubated at 37°C for 24 hours (approximately one cell cycle). Following treatment, cells were harvested for analysis or resuspended in drug free media.

2.6.2 Proteasome Inhibition Experiments

KIM-2 cells at various stages of confluency and differentiation were treated with a peptide aldehyde inhibitor of the 26S proteasome, N-carbobenzyoxyl L-leucinyl L-leucinyl leucinal (MG132). MG132 was stored as a 5mM stock in DMSO and used at a range of concentrations to inhibit proteasome function.

2.6.3 Morphological Assessment of Cell Death

KIM-2 cells were harvested as described in section 2.4.1 and cell pellets were fixed overnight in cold 70% (v/v) ethanol in PBS. The cells were then stained with the nucleic acid stain acridine orange (Molecular Probes Inc.) according to the method of Gregory (1991). Briefly, equal volumes of cells and acridine orange (5mg/ml in PBS) were mixed on a microscope slide. Green fluorescence at the wavelength 500-525nm was detected. Apoptotic cells were differentiated from viable cells by their brightly stained, condensed or fragmented nuclei and the percentage apoptotic cells was scored.

2.6.4 Flow Cytometric Analysis of Cell Death

Supernatants were removed from the cells and placed in a universal bottle, unless apoptosis was not to be analysed in this fraction. Monolayer cells were rinsed

in 1mM EDTA (v/v) in PBS. Cells were trypsinised as normal but were pipetted more vigorously to break up large clumps of cells. The cells were pelleted and resuspended in ice cold 1% FCS (v/v) in PBS and counted using a haemocytometer. 1 x 10^6 cells were placed in an eppendorf tube and centrifuged at 1000rpm for 5 minutes to pellet the cells.

The assay was performed using an Apoptosis Detection Kit (R&D Systems) which was designed to quantitatively determine the percentage of cells undergoing apoptosis. This is achieved by virtue of the ability of apoptotic cells to bind annexin V and exclude propidium Iodide (PI). Annexin V is a phospholipid binding protein, which can be used to monitor changes in cell membranes. A key characteristic of cells undergoing apoptosis is the flipping of phosphotidylserine (PS) from the inner to the outer cell membranes. This makes PS available for binding by Annexin V. Cells which are undergoing apoptosis will be bound by Annexin V, whereas necrotic cells with ruptured cell membranes will bind both Annexin V and PI, and thus apoptotic cells can be distinguished from cellular debris. Cells were resuspended in 1ml of binding buffer and 100µl of cells (1 x10^5) were aliquoted into a separate eppendorf. Cells were stained with 10µl FITC (Fluorescein isothio-cyanate) labelled AnnexinV and 10µl PI and vortexed to mix the components. The samples were incubated at room temperature in the dark for 15 minutes. 400µl of binding buffer was added to the samples before reading on a Coulter Epics XL flow cytometer at 488nm for FITC and 575nm for PI.

2.6.5 Flow Cytometric Analysis of Cell Cycle Distribution

Cells were treated as described in section 2.4.1 and fixed in cold 70% (v/v) ethanol in PBS for at least 1 hour at 4°C. 1 x 10^6 cells were rehydrated in 500µl PBS at room temperature for 5 minutes. The nucleic acid stain PI was added to the cells at 5mg/ml and samples were incubated on ice for 10 minutes. 10,000 cells were

analysed on a Coulter Epics XL flow cytometer at a fluorescence wavelength of 575nm. Cellular debris was excluded from the analysis by scatter gating.

2.7 Protein Extraction and Analysis

2.7.1 Extraction of Protein from Tissue Samples

Tissues were snap frozen in liquid nitrogen then ground to a powder, which was resuspended in 1ml RIPA$^+$ buffer. The cells were sheared to release the protein by passing through a 21 gauge needle three times. Samples were centrifuged at 13000rpm for 15 minutes at 4°C to pellet the debris. Supernatants were transferred to fresh eppendorf tubes and aliquoted prior to storage at -70°C until use.

RIPA$^+$ Buffer

50mM Tris HCl (pH7.5)

150mM NaCl

1% (v/v) Nonidet P40

0.5% (v/v) sodium deoxycholate

0.1% (v/v) SDS

2μg/ml Leupeptin

1μg/ml Pepstatin

5μg/ml Aprotinin

1mM PMSF (Phenylmethylsulphonyl fluoride)

100μM sodium orthovanadate

2.7.2 Extraction of Protein from Tissue Culture Cells

Cells were washed in ice cold 1mM sodium orthovanadate ($NaVO_3$) in PBS and then harvested from the flask using 1mM $NaVO_3$ in PBS to protect phosphate groups. Centrifugation to pellet the cells was followed by snap freezing in liquid nitrogen for storage. Otherwise the pellet was resuspended in 200µl (per T25cm^2 flask) of RIPA$^+$ buffer and extracted as above.

2.7.3 Extraction of Protein from *S. cerevisiae*

10ml cultures were grown for 48 hours at 30°C in selective media. Cells were centrifuged at 1500rpm for 5 minutes, the supernatant was aspirated and the pellet was resuspended in 500µl RIPA$^+$ buffer. Glass beads (as section 2.3.3) were added to just beneath the meniscus and the protein samples were vortexed for 30 seconds and then placed on ice for 30 seconds. This procedure was repeated for 15 minutes to lyse the yeast cells. The supernatant was transferred to a fresh eppendorf tube and centrifuged at 13000rpm for 30 minutes at 4°C to pellet the debris. The supernatant was transferred to a fresh tube, aliquoted and stored at -70°C until use.

2.7.4 Estimation of Protein Concentration

Protein concentrations were determined using the Pierce BCA detection system. The assay is based on a colour change from green to purple when protein reacts with Cu^{2+} in an alkaline medium. This gives Cu^{1+} which when complexed with the salt bicinchoninic acid (BCA) gives a purple product which can be detected at a wavelength of 570nm. The protocol involved diluting a range of bovine serum albumin (BSA) protein standards in the appropriate extraction buffer (0.2 - 2mg/ml). 10µl of the standards and 10µl of protein samples were pipetted into wells in a 96 well plate. These were diluted in 200µl of working reaction, which is composed of

50 parts Reagent A with 1 part Reagent B. Samples were mixed by rocking the plate on an orbital shaker for 30 seconds followed by a 30 minute incubation at 37°C to allow colour formation. The absorbance was read at 570nm on a Dynatech MRX plate reader. Using the BSA protein samples, a standard curve was generated which was used to determine the concentration of the test samples.

2.7.5 Denaturing (SDS/PAGE) Polyacrylamide Gel Electrophoresis

The method of Laemmli (1970) was used to perform one dimensional SDS/PAGE electrophoresis. Using a premixed 30% polyacrylamide solution (Scotlabs) a 10% or 15% resolving gel with 3% or 5% stacking gels respectively were cast. 10μg of protein sample was denatured at 99°C for 5 minutes, chilled on ice and loaded alongside prestained molecular weight markers. Gels were submerged in SDS/PAGE running buffer (25mM Tris, 192mM glycine, 0.1% w/v SDS) and run at 100 volts through the stacking gel followed by 200 volts through the resolving gel.

2.7.6 Western Blotting

Proteins were transferred by electrophoresis from the separating gel onto either a nitrocellulose filter (Schleicher & Schwell) or a polyvinylidene fluoride (PVDF) filter [Gelman Sciences] using a BioRad Protean II Transfer Apparatus. Proteins were transferred for 1 hour at 50 volts in transfer buffer (25mM Tris, 192mM Glycine, 0.01% w/v SDS, 20% v/v methanol). Following transfer the separating gel was stained with a 0.2% (v/v) Coomasie solution to ensure even loading of protein.

2.7.7 Protein Detection

Nitrocellulose filters were blocked overnight at 4°C in Tris Buffered Saline [10mM Tris HCl pH7.5, 100mM NaCl, 0.1% (v/v) polyoxyethylenesorbitan monolaurate {Tween 20}] (TBST) containing either 5% Marvel dried milk powder or BSA. Filters were incubated in primary antibody (diluted according to the manufacturer's instructions) in block solution on a shaking platform for 1 hour at room temperature. Filters were then washed for 30 minutes in TBST prior to incubation in secondary antibody (conjugated to horseradish peroxidase) diluted in TBST for 1 hour at room temperature. Filters were again washed for 30 minutes in TBST at room temperature on a rocking platform. An Enhanced Chemiluminescence (ECL) detection kit [Amersham] was used to detect the antibody-protein interaction on the membrane. ECL reagents emit light at 428nm, which can be detected by a short exposure to blue-light sensitive autoradiographic film (ECL Hyperfilm, Amersham).

2.8 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.8.1 RT-PCR Reaction for Proteasomal ATPases

RT-PCR reactions were performed using a Gibco BRL Superscript Preamplification System for First Stand cDNA sysnthesis. Single stranded cDNA was prepared by adding 5μg of day 15 gestation mammary gland total RNA to 1μl of random hexamers (50ng/μl). This mixture was denatured at 70°C for 10 minutes, chilled on ice and the following components were added:-

1μl RNAse Inhibitor

2μl 10 x PCR Buffer (200mM Tris HCl pH8.4, 500mM KCl)

2μl 25mM Magnesium Chloride ($MgCl_2$)

1µl 10mM dNTP mix (dATP, dCTP, dGTP, and dTTP)

2µl 0.1M Dithiothreitol (DTT)

The reaction was incubated at 25°C for 5 minutes before the addition of 1µl Superscript II reverse transcriptase (2U/µl) and then incubated at 25°C for a further 10 minutes. The reaction was transferred to 42°C for 50 minutes and then terminated by heat inactivation of the enzyme at 90°C for 5 minutes. The template RNA was removed from the samples by the addition of 1µl RNAse H (2U/µl) and incubated at 37°C for 20 minutes. The PCR reaction was performed using 4µl of single stranded cDNA and adding the following components:-

5µl 10 x PCR Buffer

3µl 25mM $MgCl_2$

1µl 10mM dNTP mix

1µl 10µM ATPase Primer Pairs

35µl ddH_2O

1µl Taq DNA Polymerase [5U/µl] (Boehringer)

The reactions were overlaid with 30µl mineral oil and placed in a thermal cycler and the following program run:-

94°C for 1 min

Denature at 94°C for 30 seconds
Anneal at 60°C for 1 minute
Extend at 72°C for 2 minutes
] 30 cycles

A final 5 minute extension was performed at 72°C for 1 cycle only. Negative control samples were set up for each PCR performed. These samples consisted of the above reaction performed in the absence of cDNA. The following primer pairs were used:-

TBP1 full length

5' GGGAATTCCATATGTCCACGGAAGAGATTGTC 3'

5' CTCCCCCGGGTAGAGGTGTCCCCTAGGC 3'

TBP1 Δ375

5' GGGAATTCCATATGTCCACGGAAGAGATTGTC 3'

5' GCGCCTAGGAATCTTCAGCTTTTCTGCATCCAC 3'

TBP1 Δ475

5' GGGAATTCCATATGTCCACGGAAGAGATTGTC 3'

5' GCGCCTAGGAATGTCCACCTCCATGGCCTTCAC 3'

TBP1 Δ525

5' GGGAATTCCATATGTCCACGGAAGAGATTGTC 3'

5' GCGCCTAGGGATCTCCTGGATATGCTTGTCCAG 3'

TBP1 PAS

5' TTCCATCTGTCCACGGAAGAGATTGTC 3'

5' CTGGTCGACTAGAGGTGTCCCCTAGGC 3'

TBP1 PACT

5' CCGGAATTCGCTCAAGATGTCCACGGA 3'

5' CTGGTCGACTAGAGGTGTCCCCTAGGC 3'

TBP7 PAS & PACT

5' ATGCCATGGTGGTCACTATGGAGGAG 3'

5' CTGCTCGAGCTCACTTGTAAAACTCATG 3'

SUG2 PAS

5' TTCCATATGGCGGACCCTAGAGATAAG 3'

5' CCGCTCGAGTTACACAGGTTTGTAGTCCAA 3'

SUG2 PACT

5' CCGGAATTCTCATGGCGGACCCTAGAG 3'

5' CCGCTCGAGTTACACAGGTTTGTAGTCCAA 3'

10µl of the PCR reaction was electrophoresed on a 1% TAE gel (section 2.1.9) to check a product of the correct size was present. The remainder of the reaction was run on a low melting point gel and the band of interest purified as described in section 2.1.2.

2.8.2 Differential Display RT-PCR

5µg of total RNA was used as template to prepare single stranded cDNA using a First Strand cDNA Kit (Pharmacia). Briefly, RNA was diluted to 5µl in sterile ddH_2O, denatured at 65°C for 10 minutes and the following components were added:-

1µl DTT (200mM)

4µl 8µM Oligo $dT_{12}MN$ primer

5µl Bulk Strand Mix (M-MuLV reverse transcriptase, RNAguard, dNTP mix)

The cDNA reactions were incubated at 37°C for 1 hour, followed by heat inactivation of the reverse transcriptase at 95°C for 10 minutes. cDNAs were diluted to 100µl and mixed. 10µl of the diluted cDNA reaction was further diluted in 90µl ddH_2O. This dilute cDNA stock was used in the following PCR reaction.

10µl cDNA (approximately 50ng)

2µl 20µM dNTP mix

2µl 8µM Oligo $dT_{12}MN$ primer

2µl 5µM Random 10mer primer

2µl 10 x PCR Buffer

1µl $\alpha^{35}S$ dATP (Amersham, specific activity 1000Ci/mmol)

0.3µl Taq DNA Polymerase (Boehringer, 5U/µl)

0.7µl ddH_2O

The reactions were overlaid with 30µl mineral oil and placed in a thermal cycler and amplified using the following conditions:-

94°C for 2 minutes
94°C for 30seconds
40°C for 2 minutes
72°C for 30seconds
72°C for 5minutes

40 cycles

The following primers were used:-

Oligo $dT_{12}MC$ is an equal mix of $T_{12}AC$, $T_{12}CC$, and $T_{12}GC$

Oligo $dT_{12}MA$ is an equal mix of $T_{12}AA$, $T_{12}CA$, and $T_{12}GA$

Oligo $dT_{12}MG$ is an equal mix of $T_{12}AG$, $T_{12}CG$, and $T_{12}GG$

Sox	AGTCAGTCTG
Rand 1	GTTCATAAGC
Rand 5	ACCCCGGTCA
Rand 12	ACGTCTATAG
Rand 14	TTCGGCTTGA
Rand 15	CTGATCGGAC
Rand 16	GATACCCTGT

Native 6% polyacrylamide gels in 1 x TBE buffer were prepared for differential display reactions. 8µl of the PCR reaction was loaded following dilution in loading dye, and the gel run at 250 volts overnight at room temperature. The gel was dried at 80°C for 1 hour and exposed to film overnight. When labelled PCR reactions for screening slot blots were required, $\alpha^{35}S$ dATP was substituted with $\alpha^{32}P$ dCTP and generally a mix of primer pairs were included in the reaction. Otherwise the reaction was identical to that described above.

2.8.3 Band Excision

Using the developed autoradiograph, scores were made in the film using a sterile scalpel blade on either end of the band to be excised. The autoradiograph was

overlaid on the dried display gel and used as a template to score the area of interest on the gel. This band was subsequently cut from the gel and rehydrated in 100µl ddH_2O at room temperature for 15 minutes. The eluted DNA was denatured at 99°C for 15 minutes prior to the addition of 2.5µl 20mg/ml glycogen, 1/10 volume of 3M NaAc and 3 volumes of ethanol. The DNA was stored at –20°C overnight and precipitated by centrifugation at 13000rpm for 30 minutes. DNA pellets were washed in 70% ethanol air dried and resuspended in 10µl ddH_2O. The DNA was then reamplified using the same primer combination as in the original amplification procedure. The reaction was performed as follows:-

4µl DNA

4µl 10 x PCR Buffer

2.5µl 20µM Oligo dT Primer

2.5µl 20µM Random Primer

3.2µl 10mM dNTP mix

0.3µl Taq DNA Polymerase

ddH_2O to 40µl, and samples were overlaid with 30µl mineral oil.

The reactions were transferred to a thermal cycler and amplified using identical conditions to the initial PCR reaction. One quarter of the reamplified DNA was run on a 1% agarose gel to check the reactions were successful, and the remaining PCR reaction was purified using a Qiagen PCR Purification Kit (as per manufacturer's instructions) and cloned into an Invitrogen TA (PCR) cloning vector as described in section 2.1.5.

2.9 cDNA Library Screening

2.9.1 Plating of cDNA Library

A day 15 gestation mammary gland cDNA library in Lambda Uni-Zap XR (Stratagene) provided by Dr Christine Watson, was used according to the supplier's

instructions. The strains supplied were:- XL1-Blue MRF' {genotype Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F' proAB, $lacI^qZ\Delta M15$, $Tn10(tet^r)$]} and SOLR strain {genotype e14-(mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::$Tn5(kan^r)$, uvrC, lac, gyrA96, relA1, thi-1, endA1, λR [F' proAB,$lacI^qZ\Delta M15$] Su^-(nonsuppressing)}.

50ml cultures of LB broth supplemented with 0.2% (v/v) maltose and 10mM Magnesium sulphate ($MgSO_4$) were inoculated with a single XL1-blue MRF' colony and grown overnight at 30°C in a shaking incubator. Cells were centrifuged at 2000rpm for 10 minutes and the pellet resuspended in 10mM $MgSO_4$ such that OD_{600} was equal to 0.5. These cells were stored at 4°C for a maximum of 3 days. To titre the library, serial dilutions of the phage stock were made in SM Buffer (i.e. 10^{-3} to 10^{-8} dilutions). 1μl of this diluted phage was added to 200μl of the plating strain (XL1-blue MRF' cells, OD_{600}=0.5). These reactions were incubated at 37°C, shaking for 15 minutes to allow the phage to adhere to the bacteria. 3mls of Top Agar (pre-warmed to 48°C) was added to the phage/bacteria solution and vortexed prior to pouring onto 90mm NZY plates. Plates were left at room temperature for 15 minutes to allow the Top Agar to set and then incubated at 37°C overnight. The number of plaque forming units (pfu) was subsequently counted to determine the titre of the library.

LB Broth (per litre)
10g (w/v) NaCl
10g (w/v) bactotryptone (Difco)
5g (w/v) yeast extract (Difco)
Adjust pH to 7.0 with NaOH

NZY Broth (per litre)
5g (w/v) NaCl
2g (w/v) $MgSO_4.7H_2O$
5g (w/v) yeast extract
10g (w/v) NZ amine (Difco)

LB Plates (per litre)
LB broth with 20g (w/v) bactoagar

NZY Plates (per litre)
NZY broth with 15g (w/v) Difcoagar

SM Buffer (per litre)

5.8g (w/v) NaCl

2g (w/v) $MgSO_4.H_2O$

50mls (v/v) 1M Tris HCl pH7.5

5mls 2% (w/v) gelatin

NZY Top Agar (per litre)

NZY broth with 0.7% (w/v) agarose

10 x Pipes Buffer (per litre)

4.0M NaCl

0.1M Pipes (pH6.5)

2.9.2 DNA Screening Protocol

For primary screens, phage were plated out at a concentration of 50,000pfu per 150mm NZY plate. 5 x 10^4pfu were added to 600µl plating cells (OD_{600} = 0.5) and incubated at 37°C for 15 minutes prior to the addition of 6.5mls Top Agar. This mixture was poured onto 150mm NZY plates and incubated at 37°C overnight. Plates were subsequently refrigerated at 4°C for 2 hours to chill, which prevented the Top Agar sticking to the nylon membrane (Hybond-N, Amersham). The phage were overlaid with nylon filters for 2 minutes to allow transfer of the plaques, and the filter pricked with a needle to orientate. Following transfer, the filters were denatured by submerging in a solution containing 1.5M NaCl, 0.5M NaOH for 3 minutes. Neutralisation for 5 minutes followed in a solution containing 1.5M NaCl and 0.5M Tris HCl pH8.0. Filters were rinsed for 30 seconds in 0.2M Tris pH7.5 and 2 x SSC Buffer, prior to blotting dry on 3mm paper (Whatman). Duplicate lifts were taken from each plate and performed as above, with the exception that the second filter was left in contact with the plaques for 5 minutes. DNA was fixed onto the filters by baking at 80°C for 2 hours. The agar stock plates were stored at 4°C following the transfer.

Prehybridisation buffer {2 x Pipes buffer, 50% (v/v) deionised formamide, 0.5% (w/v) SDS} was pre-warmed to 50°C prior to the addition of denatured salmon sperm DNA at 100µg/ml. Filters were incubated in 50mls of prehybridsation buffer at 42°C for 2 hours. A blank nylon filter was hybridised with the phage containing

filters to determine background hybridisation levels. Double stranded DNA probes were labelled using a Stratagene Prime-It II Kit as described in section 2.3.1. Labelled DNA was denatured at 99°C for 10 minutes and added at a concentration of 1 x 10^6 cpm/ml hybridisation buffer. Filters were hybridised at 42°C overnight in a shaking water bath. Following hybridisation, filters were washed in pre-warmed 0.1 x SSC and 0.1% (w/v) SDS buffer, initially at 50°C and then at 65°C for highly homologous signals. Membranes were subsequently blotted on 3mm paper to remove the excess liquid and wrapped in Saran Wrap and exposed to film overnight at –70°C in a cassette containing intensifying screens. Autoradiographs were developed and putative positive colonies identified. False positives were eliminated, as they generally did not appear on the duplicate filters for each plate.

2.9.3 Secondary Screen

Filters and plates were orientated using the dots and needle holes generated during the transfer protocol. The area around the signal on the film was removed from the plate using the fine end of a pasteur pipette. The agar plug was placed in 1ml SM buffer containing 20µl chloroform. Samples were vortexed and agar plugs were tittered as described before. For the secondary screen an identical procedure to the primary screen was followed, with the exception that the phage were plated at 50pfu per 90mm NZY plate, using 200µl plating cells. The plaque lifts and hybridisation procedures were identical to above.

2.9.4 *In Vivo* Excision

Rather than performing a tertiary screen, putative positive colonies from the second round screen were excised into pBluescript and a plasmid Southern blot was performed as described in section 2.1.10. This option was chosen as individual plaques were picked at the end of the second round screen. The excision protocol

allows efficient excision of the pBluescript phagemid from the Lambda Zap vector. Overnight cultures of both XL1-blue MRF' cells and SOLR cells (grown in LB broth only) were grown at 30°C in a shaking incubator. 1/100 dilutions of both 50ml cultures were made and grown at 37°C for 2-3 hours until mid-log phase (OD_{600} = 0.2-0.5). XL1-MRF' cells were centrifuged and resuspended such that OD_{600} = 1.0. SOLR cells were grown until OD_{600} = 0.5-1.0 and resuspended in 10mM $MgSO_4$ at OD_{600} = 1.0. The following components were added to a 50ml conical tube:-

200µl of OD_{600} = 1.0 XL1-MRF'cells

250µl of phage stock (>10^5 phage particles)

1µl of ExAssist helper phage (>1 x 10^8 pfu/ml)

This mixture was incubated at 37°C for 15 minutes, prior to the addition of 3mls LB broth. A further 2.5 hours incubation at 37°C with shaking followed. Cells were centrifuged at 3000rpm for 15 minutes and the supernatant transferred to a fresh tube. The supernatant was subsequently incubated at 70°C for 15 minutes and centrifuged at 3000rpm for 15 minutes. This supernatant contained excised phagemid pBluescript packaged as filamentous phage particles, which were stable at 4°C for up to 1 month. 200µl SOLR cells were added to 100µl of the above supernatant and incubated at 37°C for 15 minutes prior to plating 50µl of this reaction on LB-ampicillin (50µg/ml) plates and incubating at 37°C overnight. Colonies appearing on these plates contain pBluescript double stranded phagemid with the cloned DNA insert. Helper phage could not grow on these plates as they were unable to replicate in Su^- (nonsuppressing) SOLR strains, and do not contain ampicillin-resistance genes. Single colonies were picked into 2ml LB-ampicillin cultures and grown at 37°C overnight with shaking. Plasmid DNA was purified as described in section 2.1.6. As the cDNA in this library was directionally cloned, the restriction enzymes EcoRI and XhoI were used to cut the cDNA fragment from the vector and DNA was electrophoresed, transferred and hybridised as described in section 2.3.2. Double stranded DNA sequencing (section 2.1.11) was performed to identify the clones isolated from the library.

2.10 Two Hybrid Analysis

The genotype of *S. cerevisiae* strain Y190 used for two hybrid analysis was [MAT**a** gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 + URA3::GAL→lacZ, LYS2::GAL→HIS3 cyh^r].

Y190 pre-cultures were grown in 20mls YPD, containing Adenine Sulphate (2mg/ml), over 48 hours at 30°C. 100µl of this pre-culture was diluted in 100mls YPD containing adenine sulphate and grown until OD_{600} = 0.6-1.0. Cells were pelleted at 2500rpm for 5 minutes, and washed in 20mls ddH_20. A 5 minute spin at 2500rpm was followed by a second ddH_20 wash. The cell pellet was resuspended in 1ml ddH_20 and centrifuged at 13000rpm for 5 minutes. The supernatant was removed and the pellet washed twice in Lithium Acetate/Tris EDTA (LiAc/TE). After the final spin, the pellet was resuspended in a final volume of 1ml LiAc/TE. 50µl of Y190 cells were incubated with 1µg of each test set of DNA's with 50µg of salmon sperm DNA. Following mixing, 300µl of 40% PEG in LiAc/TE was added to the cell/DNA mixture and the reaction incubated at 30°C for 30 minutes. A heat shock at 42°C for 20 minutes was performed and the reaction was then centrifuged to remove the PEG from the samples. Cell pellets were resuspended in 1ml YPD and incubated at 30°C for 2 hours. 50µl of cells were plated out on selective media and incubated at 30°C until sufficient growth of colonies (approximately 5 days). The reactions were plated out on selective media as detailed in the following table.

Co-transformation of the test proteins, if an interaction occurs, should drive the expression of both the histidine and lacZ genes. The histidine promoter in Y190 cells is leaky therefore 3-AT is used to inhibit any residual histidine expression and thus reduce false positives. As a consequence, any colonies growing on 3-AT plates should represent interacting proteins through the expression of the histidine gene. Reactions were also plated on media containing all the necessary supplements to allow growth to check the cells had transformed efficiently. Colonies growing on 3-AT plates were picked and assayed for lacZ expression. These cells were grown on

their selective media for 4 days and then overlaid with 3mm paper. Colonies and filter paper were left in contact for 5 minutes. Immersion in liquid nitrogen fixed the colonies to the filter paper, which was then warmed to room temperature. The colony-containing filter was placed onto a piece of 3mm paper soaked in Z Buffer and the plates were incubated at 30°C until colour change was visible.

Interaction Reaction	**Media Supplements**
pACT (Activation Domain Vector) alone	Adenine, Histidine, and Tryptophan, or Adenine, Tryptophan, and 3 Amino-Triazole (3-AT) at 25mM and 50mM.
pAS (DNA Binding Domain Vector) alone	Adenine, Histidine, and Leucine, or Adenine, Histidine, and 3-AT at 25mM and 50mM.
pACT + pAS (containing test proteins)	Adenine and Histidine, or Adenine and 3-AT at 25mM and 50mM.

SC Media (per litre)
20g Agar (Difco)
20g D-Glucose (Gibco BRL)
6.7g Yeast Nitrogen Base
w/o Amino Acids (Difco)

YDP Media (per litre)
0.5% Yeast Extract
0.5% Peptone
2% Glucose

Selection Requirements
20mg/ml Adenine Sulphate
30mg/ml Leucine
30mg/ml Tryptophan
20mg/ml Histidine
25mM 3-Amino Triazole (3-AT)
50mM 3-Amino Triazole (3-AT)

Z Buffer (per litre)
16.1g $Na_2HPO_47H_2O$
5.5g $NaH_2PO_47H_2O$
0.75g KCl
0.25g $MgSO_47H_2O$
2.7ml 2-β-mercaptoethanol
1mg/ml X-gal
pH 7.0

Chapter 3:
Differential Display Analysis of Mammary Gland Development

3.1 Introduction

The mammary gland undergoes distinct developmental changes that can be divided into the following stages; fetal, postnatal, postpubertal, and adult and these are described in section 1.2. The most dramatic changes to the gland occur during pregnancy when the gland undergoes cycles of proliferation and differentiation of the epithelial component. The sequential activation of milk protein genes has been used as a marker to define the distinct stages of cellular differentiation that occur during pregnancy (Robinson *et al.*, 1995). Much of the research performed on the mammary gland has focussed on the control of milk protein gene expression, in particular on the action of prolactin in this process. It is known that prolactin activates Stat5a and 5b, closely related transcription factors that bind and transactivate milk protein gene promoters. Knock-out mice are available for both of these factors and this has led to a detailed knowledge of how these proteins signal to result in functional differentiation of the mammary gland. Endocrinologists have shown that the presence of prolactin alone is not sufficient to differentiate the mammary gland. Oestrogen and progesterone are also required for ductal outgrowth (Korach, 1994) and alveolar proliferation (Lydon *et al.*, 1995) respectively. This would suggest that mammary gland development requires a complex range of components, and the correct temporal expression of these components results in functional differentiation.

The aim of this project was to analyse changes occurring in gene expression in the developing mammary gland during pregnancy. We hoped to identify genes that were responsible for the early switch from proliferation to differentiation, and also for genes controlling the terminal differentiation events in mammary epithelial cells. The major interest in mammary gland biology until recently has focussed on the regulation of milk protein gene expression. In this study we endeavoured to identify some of the genes which acted upstream in this pathway, since the genes controlling the induction of differentiation in the mammary gland remain unknown at present. Another reason to target our analysis to the genes controlling the

differentiation process in the mammary gland was the relevance of this event in susceptibility to breast cancer. It is stablished that reproductive history in combination with other factors can affect the risk of developing breast cancer e.g. early first full term pregnancy and multiple pregnancies are associated with a lower incidence of breast cancer (Boring *et al.*, 1993, De Waard and Trichopoulos, 1988, Rosner *et al.*, 1994). Nulliparity, late first full term or interrupted pregnancy are associated with a greater risk of disease development (Daling *et al.*, 1994, Newcomb *et al.*, 1996). These observations suggested that the induction of differentiation can protect from neoplastic transformation in the breast. Thus there is a fundamental need to identify the factors present during differentiation that confer a protective role against breast neoplasia. The aim of this study was to identify genes acting during the differentiation process in the mammary gland. The prime aim of the project was to characterise the role that any target genes identified played in mammary gland development.

A number of mammary epithelial cell lines were available at the start of the study. These cell lines represent valuable *in vitro* systems on which to model mammary development. The cell line chosen for this analysis was HC11 mammary epithelial cells. HC11 cells, grown on plastic with the addition of lactogenic hormones, induce expression of the milk protein β-casein (Ball *et al.*, 1988). A synergistic action of prolactin and glucocorticoid was deemed necessary for high levels of β-casein expression, which has resulted in HC11 cells being used by a variety of groups to study control of β-casein gene expression. These cells were thus thought to be a good model for the developing mammary gland.

The approach chosen to monitor changes in gene expression in the mammary gland was differential display. Liang and Pardee (1992) originally described differential display as a method to separate and clone individual messenger RNAs (mRNAs) by means of polymerase chain reaction (PCR). The basis of the technique was to analyse genes that are differentially expressed in cells under altered conditions. The general strategy involved amplifying partial cDNA sequences from subsets of mRNA populations. The 3' primers used were designed to anchor the

primer to the polyadenylate (polyA) tail present in most mRNA species, but these primers also contained two additional 3' bases. By probability these primers should recognise a subset of the mRNA population e.g. a primer such as $T_{12}CA$ will recognise all mRNAs containing GT upstream of the polyA tail. There are twelve possible different combinations of the last two 3' bases, omitting T as the penultimate base which results in this primer recognising and amplifying one twelth of the total mRNA present in the cell. The 5' primers used were only 10bp long and random in sequence. The PCR parameters chosen were of low stringency and resulted in amplification of short products (100 to 500bp long) that were resolved on a DNA sequencing gel. The PCR reactions were performed in the presence of radiolabelled dATP allowing results to be visualised as bands on an autoradiograph. This allowed any changes in gene expression to be analysed visually.

3.2 Differential Display Analysis of HC11 Mammary Epithelial Cell Lines

The HC11 samples used for the differential display analysis had been treated in the following manner: a control HC11 sample, cells treated with dexamethasone alone for 24 hours, and cells treated with dexamethasone for 24 hours and then treated with prolactin for a further 3 hours. The samples were treated in this manner as dexamethasone pre-treatment was thought to prime the cells for prolactin treatment e.g. they responded much quicker to the prolactin stimulus when treated in this manner. The short 3 hour treatment with prolactin was chosen to target genes which were involved early in the differentiation process. The initial displays (Figure 3.1) showed that very little differences were observed between these treatments, with the majority of the bands being evenly expressed in all three samples. This suggested that the samples were evenly loaded and any changes that were observed may be real.

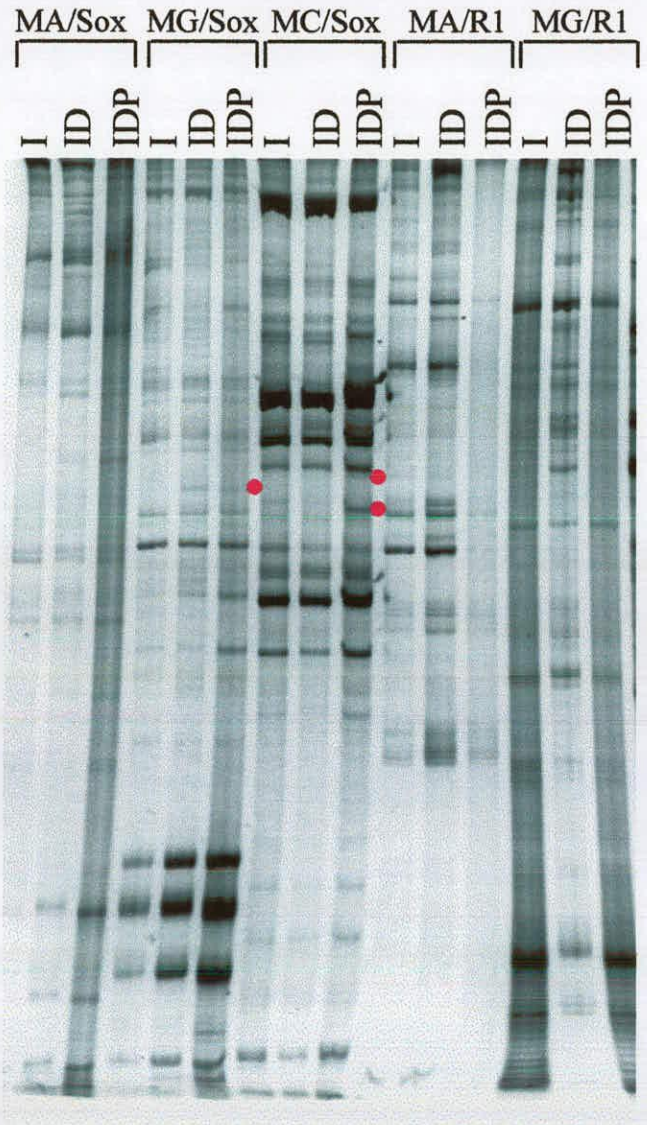


Figure 3.1 HC11 Cell Display Gel. Differential display reactions were performed on HC11 cells which had been untreated (I, Insulin), treated with Dexamethasone for 24 hours (ID) or treated with Dexamethasone for 24 hours and prolactin for a further 3 hours (IDP). The reactions containing $\alpha 35^{S}$ dATP were separated on a 5% acrylamide gel and exposed to autoradiograph overnight. MA, MG, and MC refer to the modified oligo dT primers, and Sox and R1 represent the random 10 base pair primers. The red dots represent examples of bands excised from the gels for further analysis. They are positioned to the right of the band excised and represent clones 42.1 to 42.3 shown in Figure 3.2.

Figure 3.2 shows examples of bands cut from two different display gels. These 6 bands all had expression profiles which changed with either dexamethasone treatment alone and/or prolactin treatment. The expression profiles of these transcripts are detailed in Table 3.1.

The next stage of this technique involved cloning the 3' cDNA fragment into a PCR cloning vector. This was achieved by using the autoradiograph as a template. The band of interest was scored on the autoradiograph using a scalpel blade and then the film was redeveloped to clean the surface of the film. The film was not touched with bare hands after this stage as the DNA extracted from the gel was to be subjected to a further PCR reaction. The autoradiograph was aligned with the gel using Statagene chemiluminescent markers and used as a template to score the area of interest on the dried gel. The scored fragment was cut from the dried gel and DNA subsequently eluted from the band. The DNA was precipitated in the presence of glycogen to enhance recovery. An aliquot of the recovered DNA was amplified in a similar manner as the original display reaction using the same primer combination as that which amplified the particular band. An aliquot of this reaction was electrophoresed on a 1% agarose gel to check the reamplification reaction was successful. Figure 3.3a shows the reamplified products from the 6 bands cut from the display gels in Figure 3.2. Single bands of the correct size were seen for each of these PCR products, although this was not always the case. Occasionally in these reactions we would identify smaller fragments than the cDNA fragment cut from the gel upon reamplification. When these products were cloned and sequenced they were found to represent truncated transcripts of the larger cDNA fragments. It is possible that this occurred in the reamplification reaction because the template DNA was much more limiting in the second reaction than in the original amplification reaction. This was thought to favour binding of the random 10bp primer to any sequences within the 3' cDNA with which it had a match. Closer analysis revealed that some of these truncations had only a 5 or 6bp match to the random 10bp primer. It is possible that these transcripts were not identified in the initial screen as the primer was competed for with sequences to which it had a higher homology. As these bands appear to be specific and were related to the original template cDNA,

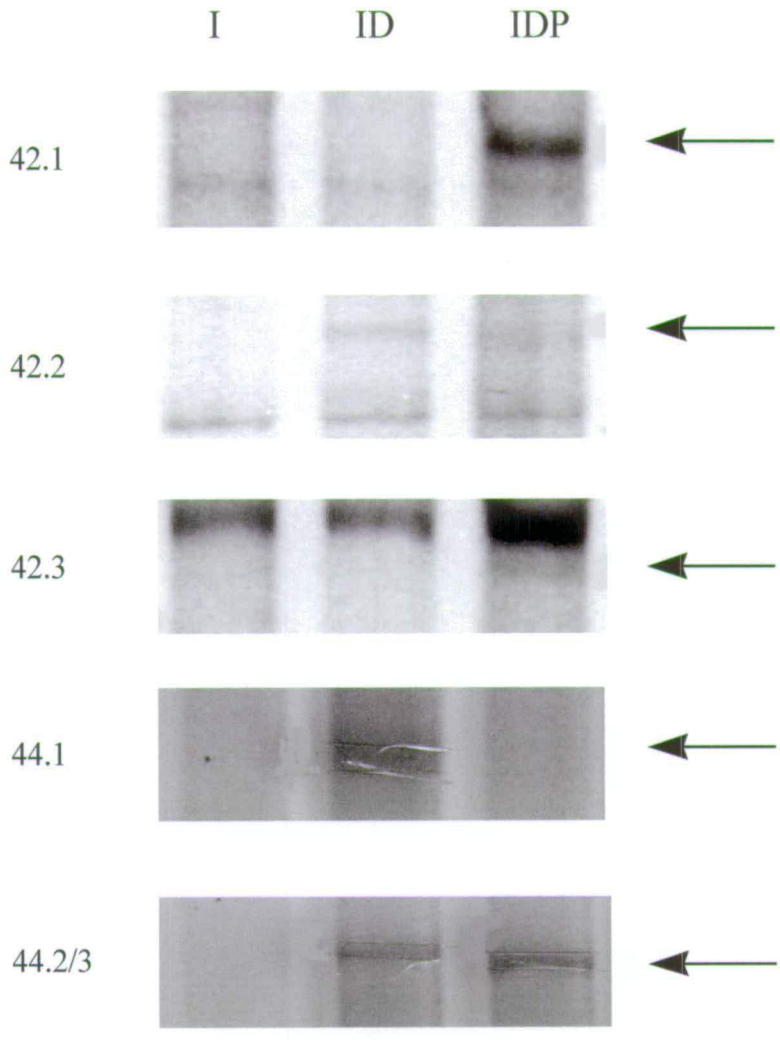
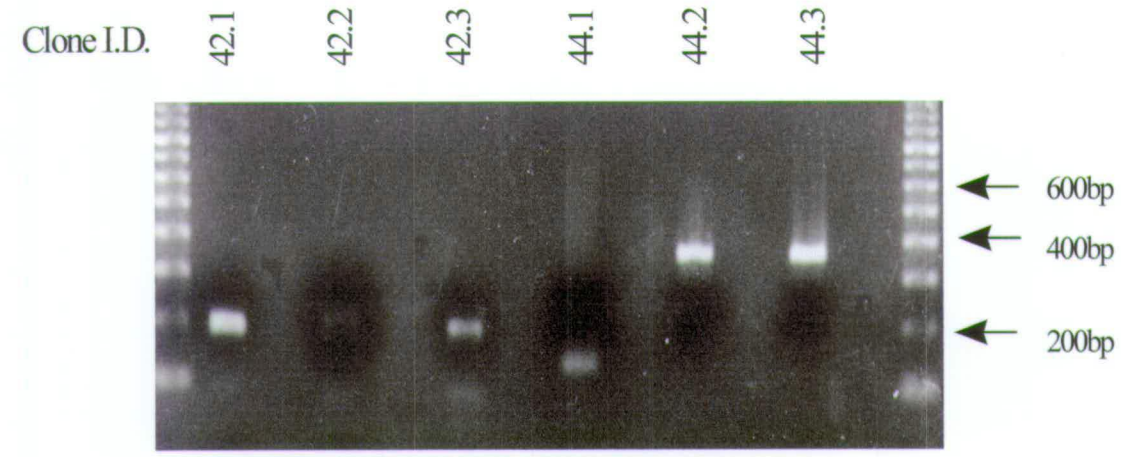


Figure 3.2 Products Cut from HC11 Display Gels. These fragments represent clones 42.1 to 44.3 which were excised from 2 separate display gels. Cells were treated with insulin alone (I) as a control, treated with dexamthasone (D) for 24 hours, or treated with dexamethasone for 24 hours followed by a 3 hour prolactin treatment (IDP). The arrows mark the position of the bands excised from the gels.

(a)



(b)

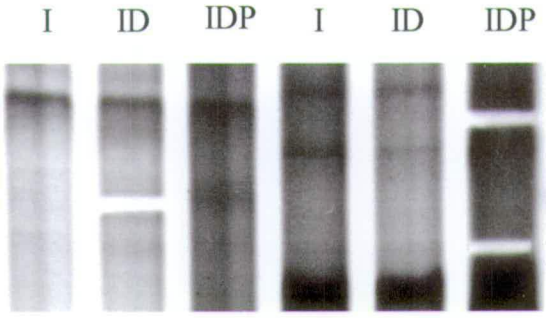


Figure 3.3 (a) Reamplification products from HC11 Displays. DNA was eluted from the dried gel fragments excised from the display gel. This DNA was subjected to a PCR reaction, which was identical to the original reaction that generated the appropriate lane on the display gel with the exception that radiolabelled nucleotide was omitted. An aliquot on the PCR reaction was subsequently run on a 1% Agarose gel to confirm the reaction was successful.
(b) Re-exposure of Display Gel following Band Excision. Following excision of dried bands from the gel a long exposure to autoradiographic film (approximately 3 days) was performed to ensure that the correct band had been excised from the gel. This gel represents clones 42.1, 42.2 and 42.3 following band excision.

therefore when they occurred they were considered not to be a problem in the reamplification procedure. The dried gel from which the bands had been excised was re-exposed to autoradiographic film to determine that the correct band and only a single band had been excised from the gel (Figure 3.3b).

The reamplified PCR reactions were purified to remove contaminating nucleotide and primers prior to ligation into a PCR cloning vector. The vector used was the TA cloning vector PCRII (Invitrogen). The PCR products were ligated into the PCRII cloning vector and transformed into competent cells. Six colonies were chosen from each PCR transformation reaction for subsequent analysis. The clones tested were cut with the restriction enzyme EcoRI, which excised the PCR product from the vector, to check the size of the products. Double stranded DNA sequencing was performed and the resultant sequence compared to sequences within the nucleotide and protein databases at BLAST (Altschul *et al.*, 1990). Results from sequence analysis are detailed in Table 3.1. Clones 42.1 and 42.3 were identified as 99% identical to the 3' 180bps of the milk protein gene β-casein. The primer combination that amplified this milk protein had been designed entirely randomly and it was not expected to identify this particular transcript. These bands had been cut from the lanes containing samples treated with prolactin and therefore β-casein, if expressed anywhere, should be in this sample. These cells were originally selected for their prolactin responsiveness, and normally by Northern Blot analysis β-casein is identified after around 4 days treatment with prolactin and dexamethasone. It was surprising to detect expression of β-casein as early as 3 hours after prolactin stimulation. This observation validates the use of this technique as we have identified a gene product documented to be differentially expressed in the mammary gland. These results also suggest that differential display is an extremely sensitive technique for detecting changes in gene expression. This particular primer pair (oligo $dT_{12}MC$ and Sox) was subsequently used as an internal control for the following display reactions.

Clone	Reamplified PCR Size (Base pairs)	Identity/ Homology	Display Expression Profile	Differential Expression Confirmation
42.1	180bp	99% Identical to 3' murine β casein	Induced by Prolactin alone	Yes (Slot blot and Northern)
42.2	210bp	77% Homologous to exons 2 and 3 of murine *c-abl* oncogene	Induced by Dex, and Prolactin	No (Slot Blot)
42.3	210bp	99% Identical to 3' murine β casein	Induced by Prolactin alone	Yes (Slot Blot and Northern)
44.1	150bp	Unknown/ Novel	Induced by Dex alone	No (undetectable by Slot Blot)
44.2	350bp	90.8% Identical to murine 5α steroid reductase	Induced by Dex and Prolactin	Yes (Slot Blot) No (Northern – expressed in all samples)
44.3	350bp	90.8% Identical to murine 5α steroid reductase	Induced by Dex and Prolactin	Yes (Slot Blot) No (Northern – expressed in all samples)

Table 3.1. Summary of Clones isolated from HC11 Differential Display Reactions. The table details the size of the PCR products, nucleotide sequence homology, original expression profile by display, and whether the expression profile was confirmed.

Band 42.2 was identified to be 77% homologous to exons 2 and 3 of murine *c-abl*. This transcript was isolated in both dexamethasone and prolactin treated samples. c-abl is a member of the non-receptor class of protein tyrosine kinases, and was originally identified as the cellular homology of *v-abl*, the transforming component of the Abelson Murine Leukaemia virus. c-abl is expressed ubiquitously in mammalian cells. It is localised in the cytoplasm in association with the cytoskeleton (on actin filaments) and also in the nucleus (McWhirter and Wang, 1991). *c-abl* contains an F actin binding site and a sequence specific DNA binding domain. This DNA binding activity is lost during mitosis due to the phosphorylation of the C-terminal segment that contains the DNA binding motif. Overexpression of *c-abl* causes G0/G1 cell cycle arrest suggesting that it acts as a negative regulator of cell growth. If this transcript represents a mammary specific factor that acts in a similar manner to c-abl, then the expression profile observed would fit with a growth arrest function. The transcript is only expressed in samples that are being induced to differentiate by the removal of EGF and the addition of dexamethasone and prolactin to the cells. The removal of EGF from the media eliminates the growth stimulus, thus the cells no longer have their proliferative signal. The addition of hormones and glucocorticoid induces differentiation and as a consequence the cells may exit the cell cycle. These events are similar to those seen when *c-abl* induces growth arrest, therefore we may have identified a gene whose action is to induce growth arrest in cells in order to allow the differentiation process to commence. Band 44.1, which was only 150bp long, had no known sequence homology.

Bands 44.2 and 44.3 were expressed and excised in the samples induced to differentiate with dexamethasone (44.2) and prolactin (44.3) and subsequently identified as being 90% identical to the steroid 5α-reductase gene. Steroid 5α-reductase is an enzyme responsible for the conversion of testosterone to the more potent dihydrotestosterone. It is a gene required for the differentiation of the external male genitalia (Anderson *et al.*, 1989). The absence of steroid 5α-reductase activity results in a rare form of male pseudohermaphroditism, where the male external genitalia differentiate as female structures (Walsh *et al.*, 1974, and Imperato-McGinley *et al.*, 1974). The expression of this enzyme is generally restricted to

androgen responsive tissues, but it has been found expressed at high levels in the female rat liver but not in male rat liver. It has subsequently been described as being expressed in the ovary (Anderson *et al.*, 1989). Steroid 5α-reductase is documented to play a role in the induction of the differentiation process in the testes. A simple explanation for the presence of this enzyme in mammary epithelial cells would be that this gene product plays a role in steroid hormone metabolism (Normington and Russell, 1992), and the mammary gland is one organ that is extremely responsive to hormonal changes. This enzyme plays a role in the metabolism of progesterone, an androgen that is highly expressed in the pregnant mammary gland. It may also be involved in the differentiation process in the mammary gland, which would correlate to the observed expression profile of this transcript.

Generally, when differential expression of a clone was identified by differential display analysis, its expression profile was confirmed by examining mRNA expression, usually by Northern Blot analysis or RNAse Protection Assay. For the six clones described above a modified slot blot analysis was performed to confirm expression profiles. Plasmid DNA was fixed on nylon membranes and hybridised with a radiolabelled PCR reaction. This PCR reaction was performed for each of the RNA samples used in the display reactions and included all the primer combinations used to generate all of the excised clones. The results of this analysis are summarised in Table 3.1. Slot Blot Analysis confirmed that the β-casein transcript was present only in the samples treated with prolactin. This result confirmed what was previously known about this transcript, that β-casein was prolactin responsive (Ball *et al.*, 1988). The expression profile observed by display for clones 42.1 and 42.3 was identical to that observed by Slot Blot analysis. Clone 42.2, with high homology to exons 2 and 3 of murine *c-abl*, was shown to be present in all three samples by Slot Blot Anlaysis. This result differed from that seen by differential display, where this transcript was originally thought to be present in only the dexamethasone and prolactin treated samples. No signal was observed for clone 44.1 by Slot Blot Analysis. In contrast, clones 44.2 and 44.3 appeared to be differentially expressed as confirmed by Slot Blot analysis. Northern hybridisation was performed on HC11 cell RNA using clone 44.3 (steroid 5α-reductase) and

showed a weak signal in all three samples. This result conflicted that observed by Slot Blot where this clone was observed to be differentially expressed.

The results on the β-casein transcript would suggest that this Slot Blot technique could be used to confirm differential expression. On further analysis it would appear that this modified Slot Blot method was only adequate for identifying genes which are relatively abundant. It was used to confirm β-casein expression correctly but this transcript accounts for around 20% of the total mRNA within the cell by late pregnancy. For transcripts of lower abundance this method was less than adequate. This may be due to the problem with the labelling technique. The method involved performing a PCR reaction containing all of the primers used to generate the bands excised from the display gels. Effectively each lane on the display gel was recreated in the labelling reaction e.g. in this case where 6 transcripts were to be analysed 4 different sets of primer combinations were used. This resulted in 4 lanes of the display gels being recreated in one reaction, which suggested that the particular transcript of interest would be a extremely small proportion of the total cDNA present. This may have resulted in difficulty in detecting transcripts that were not particularly abundant in the original starting RNA population. The Slot Blot method was chosen for an initial screen because it allowed the examination of all 6 clones in one reaction, as opposed to hybridising 6 Northern Blots. This was of importance in the display reactions as a vast number of clones can be generated very quickly and much of the time involved with this technique is that of confirming differential expression. In retrospect, it was apparent that this screening method had its limitations, and could not empirically confirm differential expression e.g. in the case of clones 44.2 and 44.3. As the relative abundance of the majority of the bands excised from the differential display gels is unknown, it may be prudent to perform Northern Analysis on these clones for a more definitive answer.

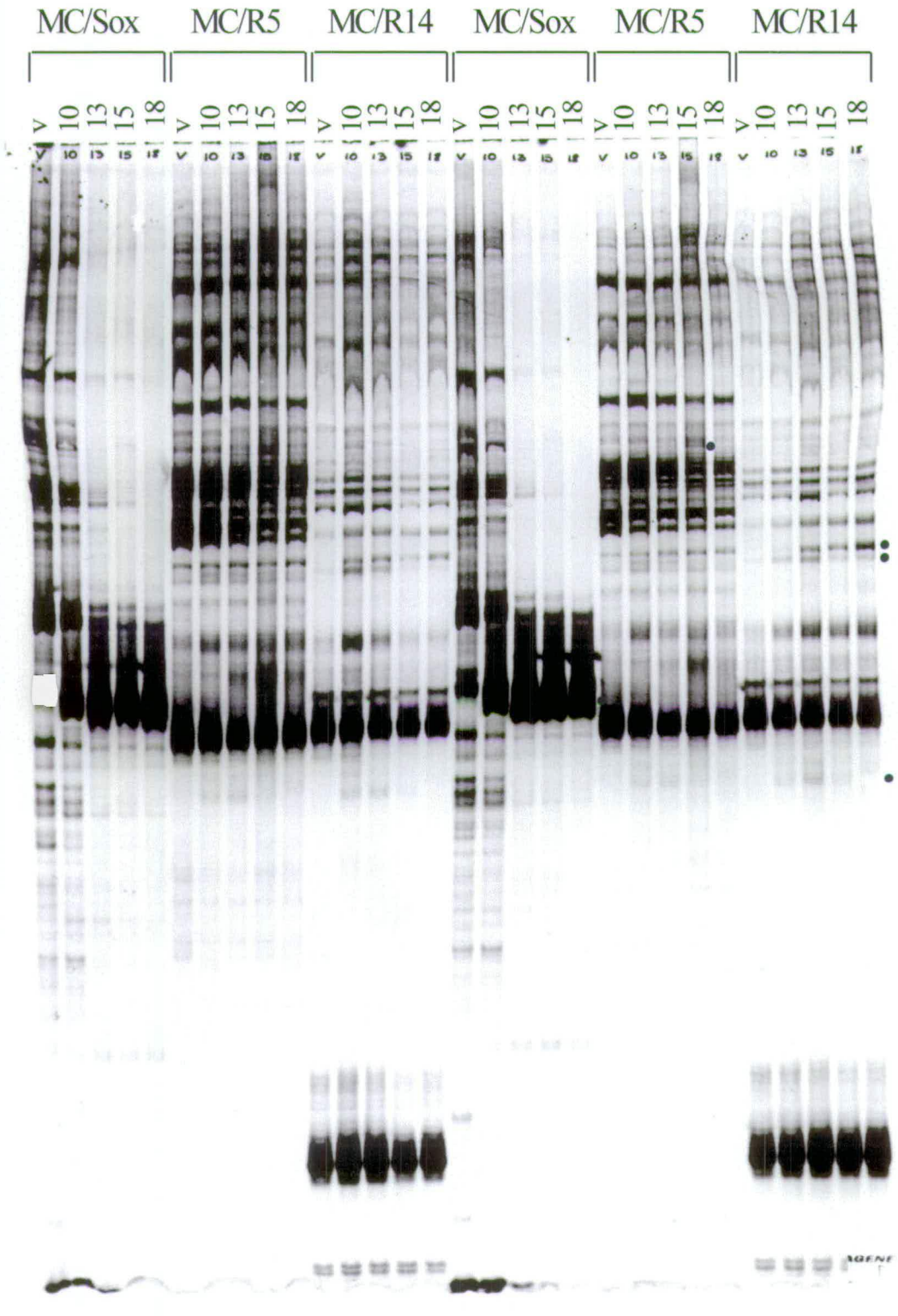
The studies using RNA from HC11 cells had revealed a small number of false positives identified in this analysis. Taking clone 42.2 as an example, we demonstrated by Slot Blot analysis that this clone was not differentially expressed. In general we exposed the differential display gels to autoradiographic film for a

maximum of 3 days. This resulted in a strong signal being observed for the vast majority of the bands present. Re-exposure of differential display gel #42 for a longer period resulted in the possibility that a very weak band may have been present in the untreated insulin sample. This observation confirmed the results obtained using the screening method. Although this transcript was present in the untreated control sample, by display and Slot Blot this appeared much weaker than that observed in the dexamethasone and prolactin treated samples. This result led to the observation that there may be some subtle changes in the expression of genes, which could be overlooked when two or three samples were compared side by side. This could be overcome when analysing a time-course of samples, where gene expression could be compared over 5 or 6 samples. This may also eliminate the problem of false positives, as any change in gene expression observed throughout 6 samples is more likely to be reproducible than changes observed between only 2 or 3 samples.

In general, a less complex system is preferred for use in differential display reactions e.g. if an *in vitro* cell culture model was available, this was the preferred template RNA for differential display as opposed to whole organs. This was thought to be due to the assumption that when using a whole tissue for analysis some differences may appear due to different populations of cells present in the tissue examined. This was the original reasoning behind using a mammary epithelial cell line as a model for differentiation changes occurring during pregnancy in the mammary gland. As a result of the initial display reactions working well in the cell lines we chose to analyse mammary gland tissue in the differential display reactions. The mammary gland is different from most other organs in that it contains only epithelial cells and stroma and in this respect should not represent a problem for use in differential display. Examining differentiation changes in the mammary gland itself was a more relevant approach to this question. Although the mammary epithelial cell lines available at this time could differentiate and express β-casein, they were unable to mimic terminal differentiation. This is marked by the expression of WAP in the mammary gland, and none of the available cell lines express this milk protein, therefore they may not be quite as relevant to mammary gland differentiation as originally assumed.

3.3 Differential Display Analysis of Murine Mammary Gland During Pregnancy.

Differential display analysis was performed on samples from virgin through to late gestation mammary glands. Mammary glands were collected from C57BL6/CBA mice at non-pregnant, day 10 gestation, day 13 gestation, day 15 gestation, and day 18 gestation time points. The strain of mice used had a gestation period of approximately 21 days. Differential display analysis was performed in an identical manner to those performed on HC11 mammary epithelial cells. Figure 3.4a shows display analysis on mammary gland samples and represents samples that were performed in duplicate. Each set of reactions was performed entirely independently and comparison of the corresponding lanes showed >95% accuracy in the reproduction of banding patterns. Resolution of these samples showed that the vast majority of the bands were common within a set of 5 samples. These observations suggested that performing the display reactions on a developing tissue would not result in changes observed which were due to changes in proportions of cell populations in the mammary gland. Figures 3.4b and 3.4c represent bands that were cut from a range of differential display gels for further analysis. All of these bands were excised and reamplified in a manner described in section 3.2. Figure 3.5 shows the results of these reamplifications. Truncated products were obtained for a range of these clones, and sequence analysis confirmed in most cases that where two PCR products were obtained these represented overlapping fragments of the same cDNAs. The reamplified PCR products were cloned into the PCRII cloning vector and the plasmid DNA from the resultant colonies sequenced. Table 3.2 summarises the data obtained on these clones.


MC/Sox
MC/R5
MC/R14
MC/Sox
MC/R5
MC/R14
V 10 13 15 18

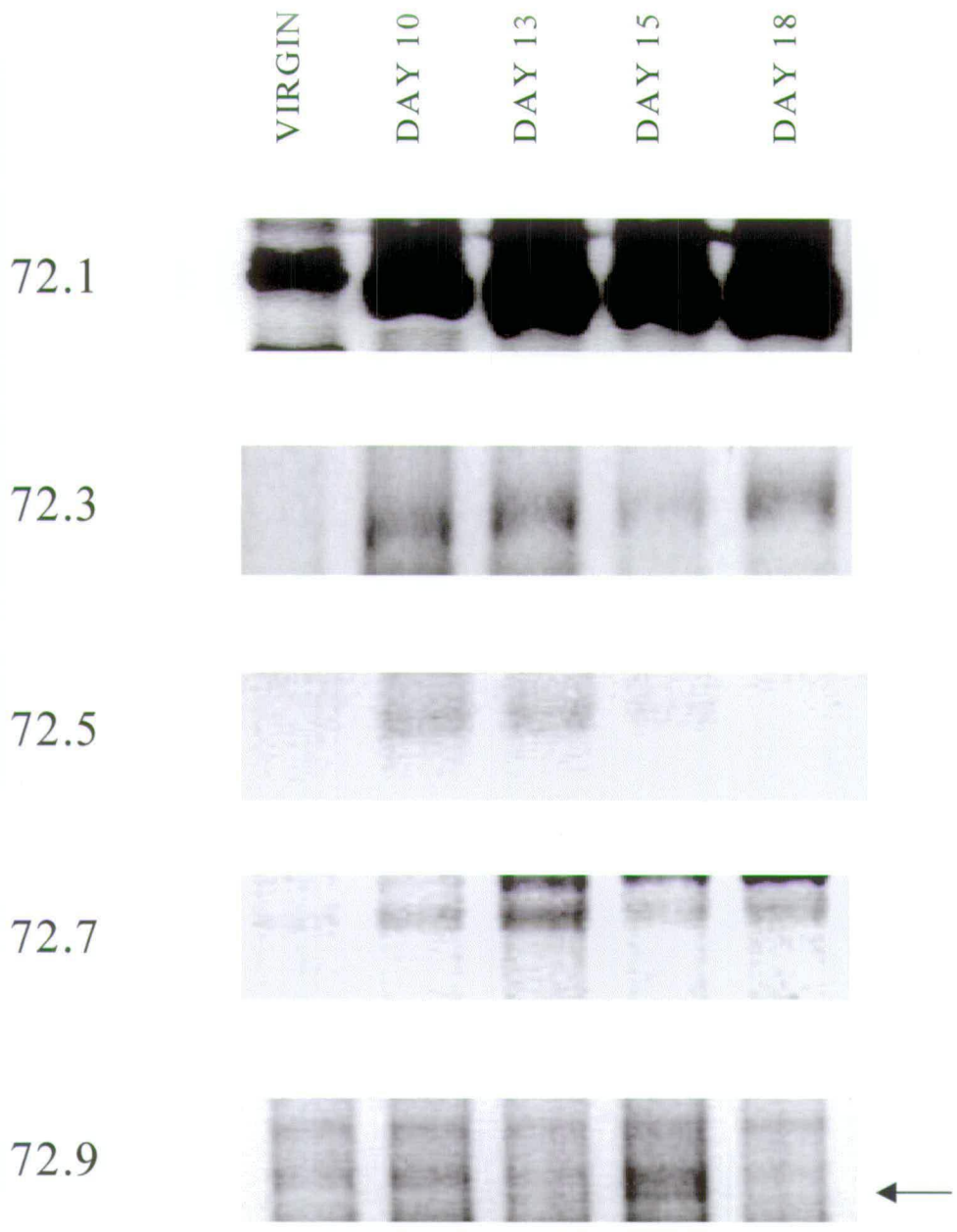


Figure 3.4b Products Cut from Mammary Gland Display Gels. These fragments represent 5 clones that were excised from the display gel shown in Figure 3.4a. The display gel was performed on cDNA extracted from the mammary glands of mice from virgin until late gestation. The arrow represents the band excised for clone 72.9, present on day 15 gestation.

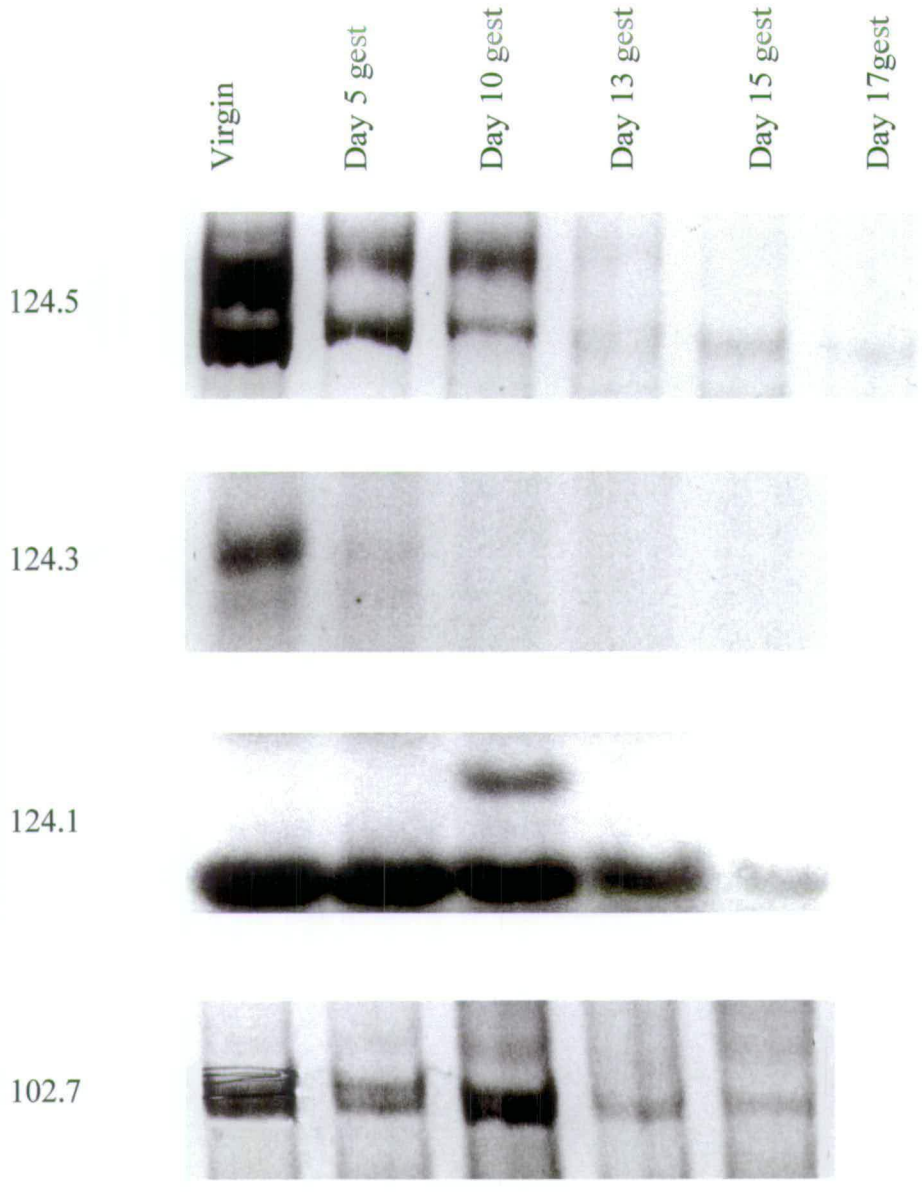


Figure 3.4c Products cut from Mammary Gland Display Gels. These fragments represent 4 clones cut from a range of display gels performed on cDNA prepared from virgin mammary glands through to day 17 gestation mammary glands. Clones 102.7 and 124.5 are expressed highly from virgin through until day 10 gestation. In both cases the upper and lower bands represent truncated products of the same cDNAs.

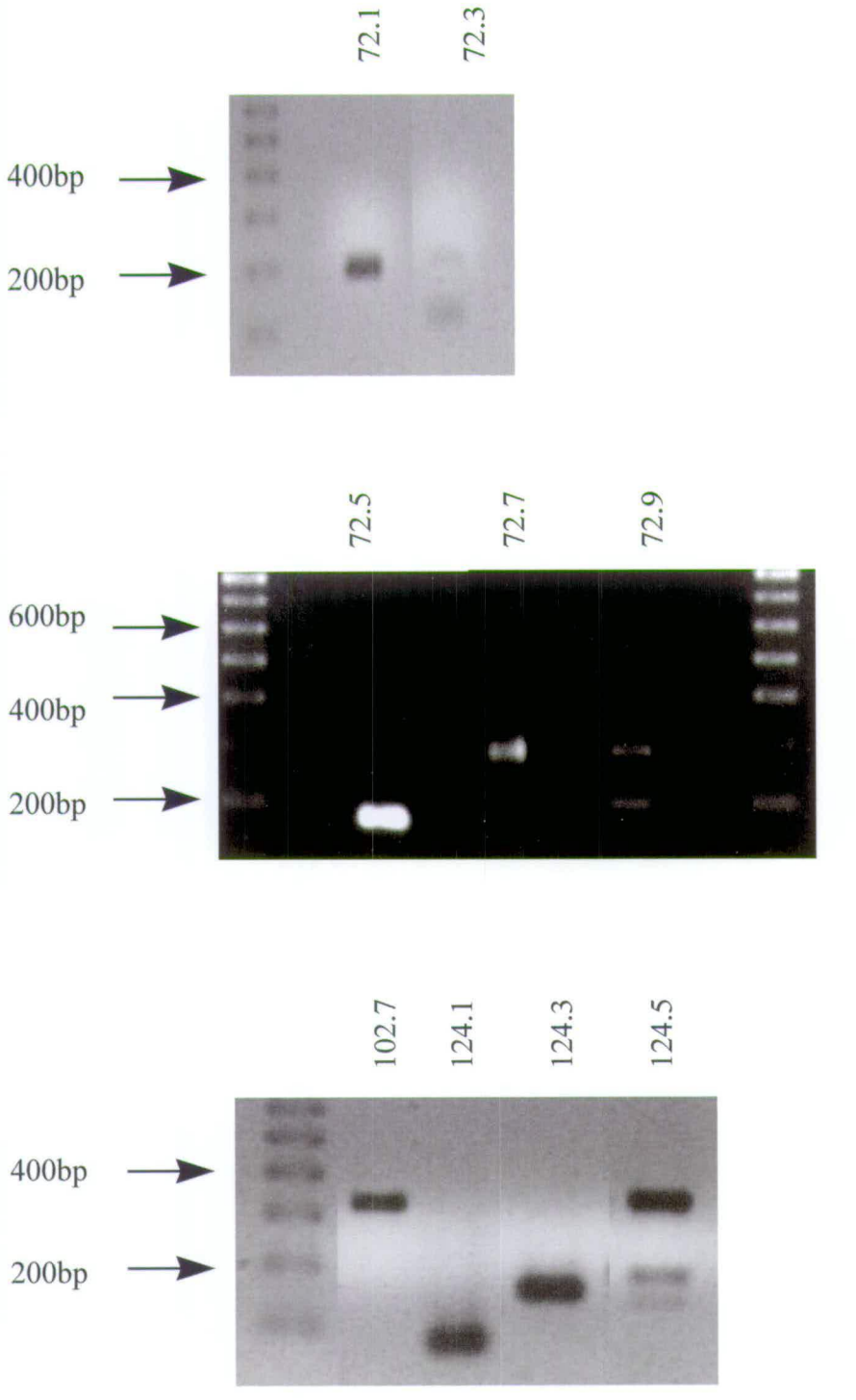


Figure 3.5 Reamplified Products from Mammary Gland Displays. DNA was eluted from the dried gel fragments and subjected to a further PCR reaction. This second reaction was identical to the original reaction which generated the appropriate lane on the display gel with the exception that radiolabelled nucleotide was omitted. An aliquot of the PCR reaction was subsequently run on a 1% Agarose gel to confirm that the reaction was successful.

Clone 72.1 was identified as 100% identical to murine β-casein. This reaction was performed as an internal control for the PCR reactions. We had previously identified β-casein using this particular primer pair (oligo $dT_{12}MC$ and Sox) in HC11 cells. The expression pattern observed for this clone, increasing gradually throughout gestation, was identical to that which we expected to see for this milk protein, and this was confirmed by Northern Blot analysis (Figure 3.6). The mammary gland begins to express β-casein from around day 10 of gestation, and this expression increases as the gland develops towards the lactation period (Ball *et al.*, 1988). We identified a band for this transcript in the control non-pregnant sample by differential display, which was not expected. Generally, virgin mammary glands are thought not to express β-casein, but it has been observed *in vitro* that embryonic mammary glands are able of responding to lactogenic hormones in culture. These glands produce a casein-like material which comigrates with authentic casein (Ceriani, 1970). It is possible that no one has previously used a method as sensitive as differential display to analyse mammary gland mRNA, hence β-casein has never been detected this early. These studies using embryonic mammary glands suggest that this result was not as strange is it had first appeared. Another reason that these mammary glands express β-casein may be due to the fact that the mice used had been previously mated, but were not pregnant. The change in circulating hormone levels caused by mating may have resulted in some small changes in the mammary gland e.g. may have acted to stimulate the gland in a way similar to an early pregnancy signal.

Clones 72.4, 72.6, and 72.8 were all excised from the same lanes as clone 72.1 (β-casein). Comparison of these primer combinations (oligo $dT_{12}MC$ and Sox) in Figure 3.4a shows that a high proportion of the bands present in these lanes appear to decrease as the gland develops during pregnancy. These three clones were chosen for further analysis to determine if the banding pattern was real or an artefact due to the high expression of β-casein in late gestation mammary gland. Sequence analysis revealed no major homologies of any of these clones with sequences in the databases searched. Northern Blot analysis revealed that expression could be detected for two of the three clones. Results from both of these clones showed that the expression

Clone 72.1 was identified as 100% identical to murine β-casein. This reaction was performed as an internal control for the PCR reactions. We had previously identified β-casein using this particular primer pair (oligo dT$_{15}$MC and Sox) in HC11 cells. The expression pattern observed for this clone, increasing gradually throughout gestation, was identical to that which we expected to see for this milk protein, and this was confirmed by Northern Blot analysis (Figure 3.6). The mammary gland begins to express β-casein from around day 10 of gestation, and this expression increases as the gland develops towards the lactation period (Ball *et al.*, 1988). We identified a band for this transcript in the control non-pregnant sample by differential display, which was not expected. Generally, virgin mammary glands are thought not to express β-casein, but it has been observed *in vivo* that embryonic mammary glands are able of responding to lactogenic hormones in culture. These glands produce a casein-like material which comigrates with authentic casein (Cerini, 1970). It is possible that no one has previously used a method as sensitive as differential display to analyse mammary gland mRNA, hence β-casein has never been detected this early. These studies using embryonic mammary glands suggest that this result was not as strange is it had first appeared. Another reason that these mammary glands express β-casein may be due to the fact that the mice used had been previously mated, but were not pregnant. The change in circulating hormone levels caused by mating may have resulted in some small changes in the mammary gland e.g. may have acted to stimulate the gland in a way similar to an early pregnancy signal

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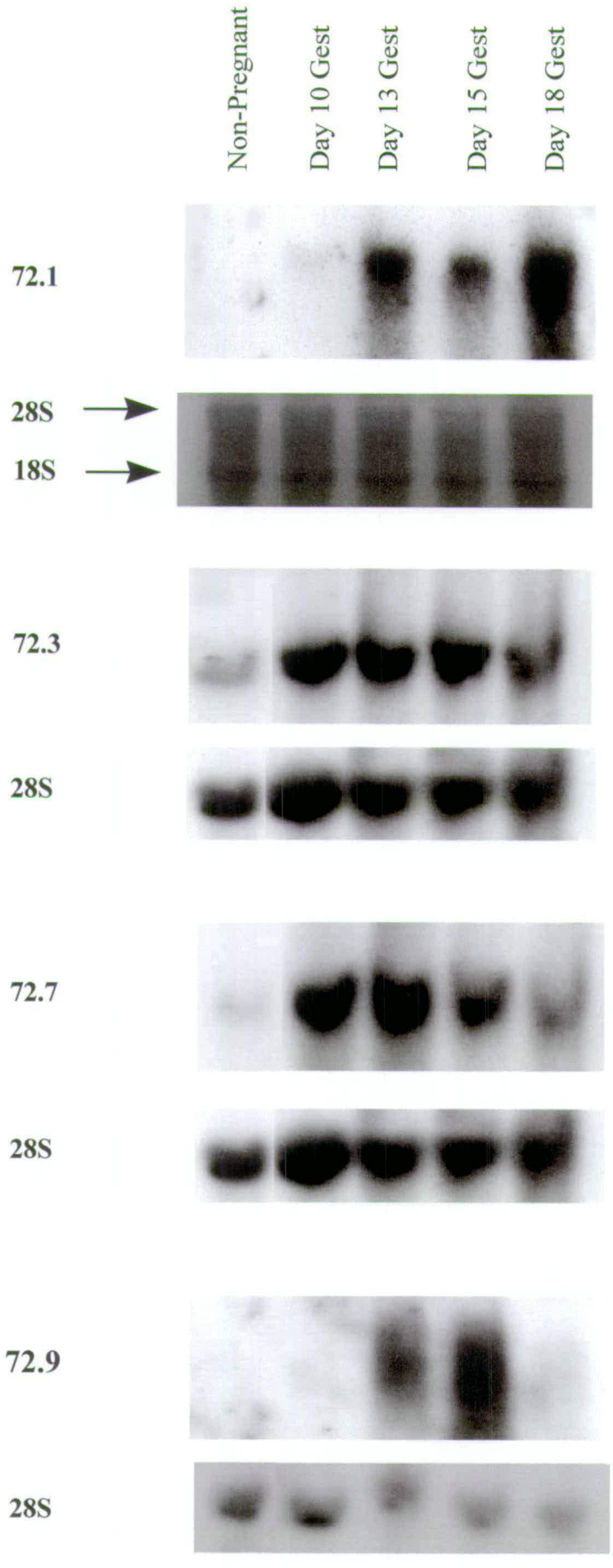
Figure 3.6 mRNA Expression of Clones isolated from Mammary Gland Display Gels. Total RNA was isolated from the mammary glands of C57/CBA mice at various stages of gestation e.g. non-pregnant represent that which were mated but failed to become pregnant, or day 10, 13, 15 or 18 of gestation. The strain of mice used had a 21 day gestation period. 10μg RNA was electrophoresed prior to blotting on nylon membranes. Each membrane was hybridised against radiolabelled cDNA prepared from the excised bands from the display gels. Membranes were washed and exposed to autoradiographic film prior to being hybridised with an oligonucleotide against 28S ribosomal RNA to allow quantitation. The second panel, which is the loading control for clone 72.1, represents the 18 and 28S RNA stained with ethidium bromide prior to RNA transfer onto membrane.


Non-Pregnant
Day 10 Gest
Day 13 Gest
Day 15 Gest
Day 18 Gest
72.1
28S
18S
72.3
28S
72.7
28S
72.9
28S

Clone	Reamplified PCR Product Size	Identity/ Homology	Display Expression Profile	Differential Expression Confirmation
72.1	180bp	100% Identical to 3'murine β-casein	Increase from day 10 onwards	Yes (Northern)
72.3	225bp	99% Identical to murine TBP1	Increase from day 10 onwards	Yes (Northern)
72.4	175bp	Unknown/ Novel	Increased up to day 10 then decreased	No (Northern)
72.5	175bp	Unknown/ Novel	Increased on days 10 and 13	Yes (RPA)
72.6	200bp	Unknown/ Novel	Increased up to day 10 then decreased	No (Undetectable)
72.7	250bp	Unknown/ Novel	Increased on days 10 and 13	Yes (Northern)
72.8	250bp	Unknown/ Novel	Increased up to day 10 then decreased	No (Northern)
72.9	275bp	Unknown/ Novel	Increased on day 13, peaks on day 15, then off in day 18	Yes (Northern)
102.7	250bp	Unknown/ Novel	Increased in virgin, and days 5 and 10	No (Undetectable)
124.1	100bp	Unknown/ Novel	Present on day 10 only	No (Undetectable)
124.3	150bp	Unknown/ Novel	Present in virgin only	No (Undetectable)
124.5	250bp	Unknown/ Novel	Increased in virgin, and days 5 and 10	No (Undetectable)

Table 3.2 Summary of Clones Identified in Mammary Gland Differential Display Reactions. The table summaries the clones excised, their size, expression profiles, and whether they were confirmed to be differentially expressed.

profile was constant throughout gestation, in conflict with the pattern observed on the display gel. These results can be explained by the fact that β-casein represents around 15-20% of all the mRNA present in the cell by late gestation. As this transcript is so abundant, it was assumed that β-casein was binding the majority of the primer present in the mid to late gestation samples resulting in what appeared to be a decrease in a high proportion of transcripts in these particular samples.

Clone 72.3 had an expression profile by differential display that appeared to increase in expression from day 10 gestation onwards as the gland develops during pregnancy. Sequencing revealed this clone to be the mouse homologue of Tat Binding protein 1 (TBP1). TBP1 was originally identified as a protein that interacts with the Human Immunodeficiency Virus (HIV) Tat Transcactivator (Nelbock *et al.*, 1990). TBP1 was described as binding to HIV Tat and specifically repressing Tat mediated transactivation. More recently, TBP1 has been described as a member of a family of Mg^{2+} dependent ATPases, which play a role in capturing ubiquitinated proteins targeted for degradation (Dubiel *et al.*, 1992). These ATPases function as part of the 26S proteasome and yeast mutation studies have shown that expression of one of these proteins is essential for cell cycle progression (Gordon *et al.*, 1993). The expression profile for clone 72.3 was confirmed by Northern Blot analysis (Figure 3.6) and showed a ten-fold increase in expression by mid gestation. This result paralleled that observed by differential display.

Clone 72.7, which peaked in expression on days 10 and 13 gestation, had no known sequence homology when compared to the databases. This expression profile was confirmed by Northern Blot analysis (Figure 3.6). Clone 72.9, present on day 13 and peaking on day 15 gestation, again showed no identifiable sequence homology. Northern Blot analysis confirmed the expression profile observed by differential display for clone 72.9 (Figure 3.6).

Clone 72.5 had an expression profile that peaked on days 10 and 13 of gestation. No sequence homology was obtained for this clone. Northern Blot analysis was performed using this 3'cDNA, and no signal was observed. As a more

sensitive method for detecting mRNA expression, an RNAse Protection Assay (RPA) was performed. To optimise the system, we performed an RPA with clone 72.1 (β-casein) as the expression profile of this clone was well known. Results showed the correct expression profile for β-casein (Figure 3.7a) and thus the assay was performed using clone 72.5 (Figure 3.7b). The RPA confirmed a similar pattern to that observed on the differential display gel, with high expression on days 10 and 13 gestation.

None of the clones represented by 3' cDNA fragments 102.7, 124.1, 124.3, and 124.5 had any identifiable sequence homology when screened against both nucleotide and protein databases. Clone 124.1 appeared to be represented by a single PCR reamplification product, but when sequenced revealed that there was an equal proportion of three different cDNAs present. Although there are reports that one band represents one gene in these display gels (Guimarares *et al.*, 1995), other groups have reported that one band may represent additive products of distinct cDNA fragments (Li *et al.*, 1994, Callard *et al.*, 1994). Approximately 20 individual clones were sequenced for clone 124.1 and it became apparent that none of the three transcripts identified were represented more frequently than any of the others. Northern Blot analysis was performed for these three cDNAs as well the other clones and no signal was detected for any of these excised bands. RPAs were subsequently performed for each of these clones, again yielding no discernible results. The detection problems were not as a result of the quality of the template RNA used in these reactions as 18S ribosomal RNA probes were used for both Northern and RPA analysis and these produced signals. In order to confirm whether the banding patterns we observed represented PCR artefacts, we prepared fresh RNA samples and repeated the differential display reactions. Identical results were obtained from this second display gel suggesting that these bands did indeed represent differentially expressed cDNAs, which are probably of extremely low abundance. More recently, polyA$^+$ RNA was prepared from mammary epithelial cells that were induced to proliferate, differentiate, or undergo apoptosis. Northern Blot analysis revealed no signals for any of these clones.

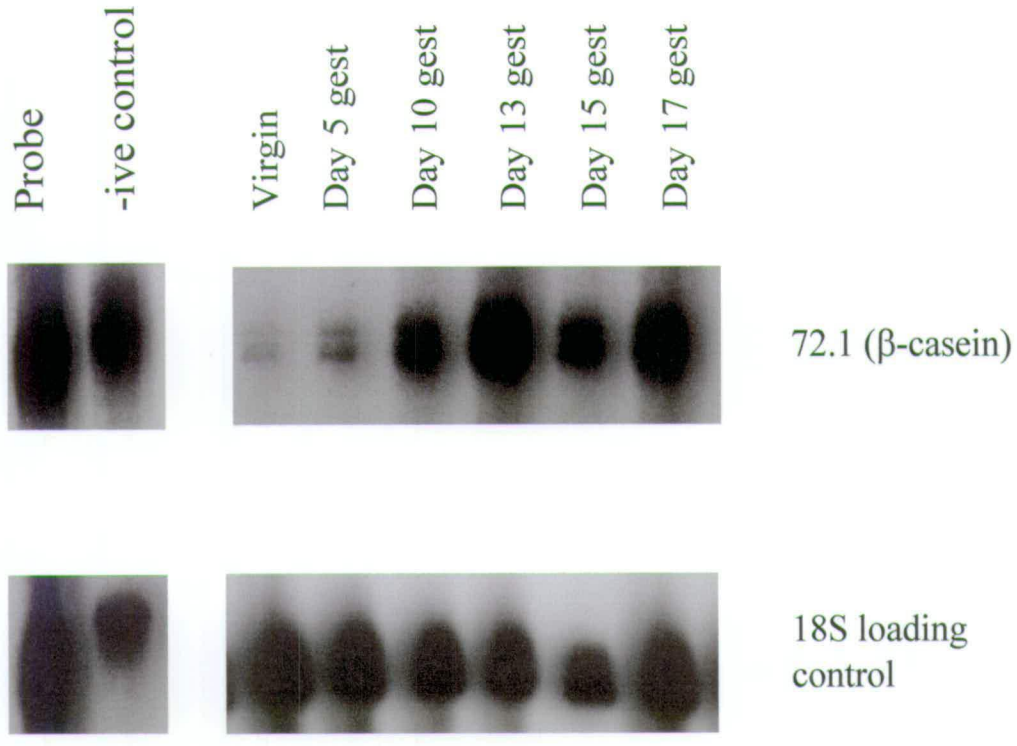


Figure 3.7a Optimisation of RNase Protection Assay. An RNase Protection Assay was performed using clone 72.1 (β-casein) to optimise the system for use with mammary gland RNA. Sense and anti-sense transcripts were generated from clone 72.1 and hybridised to RNA from mice at different stages of gestation (gest). The same samples were also hybridised with an oligonucleotide generated against 18S RNA as a loading control. The reactions were performed in the presence of ^{32}P dUTP and exposed to autoradiographic film overnight. The control samples are represented by probe alone and undigested yeast RNA hybridised to clone 72.1 (-ive control). These control samples are represented by a 3 hour exposure to autoradiographic film.

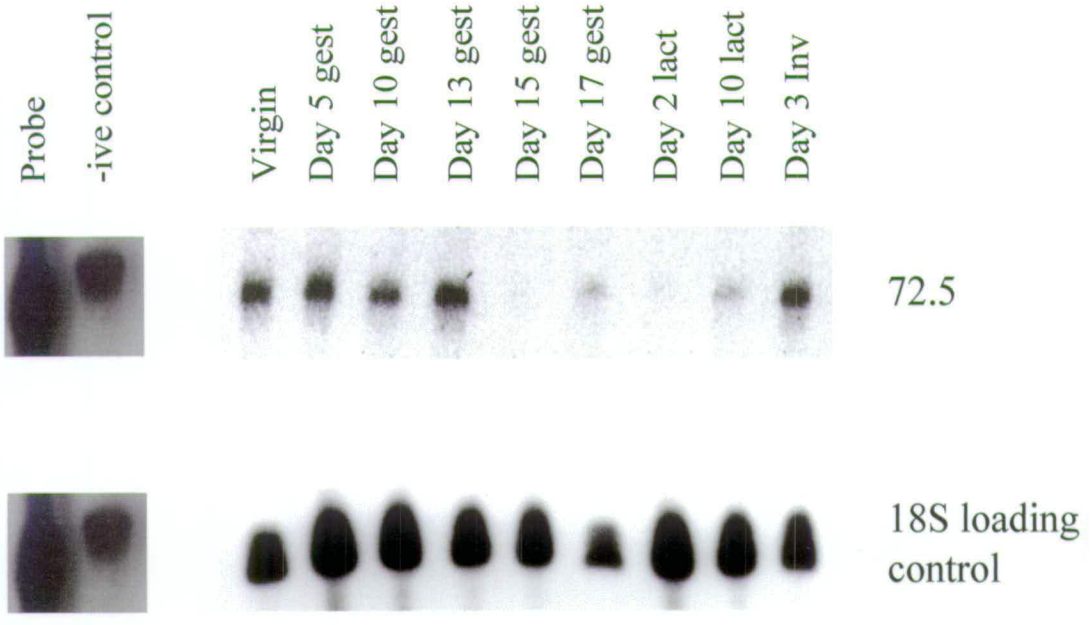


Figure 3.7b Expression Profile of clone 72.5. An RNase Protection Assay was performed on clone 72.5 (~200bp long) as no signal had been detected by Northern hybridisation. Sense and anti-sense transcripts were hybridised to RNA from the mammary gland of mice at different stages of gestation (gest), lactation (lact), and involution (Inv). Reactions were performed in the presence of ^{32}P-dUTP and exposed to autoradiographic film overnight. The control samples are represented by probe alone and undigested yeast RNA hybridised with clone 72.5 (-ive control). These samples were exposed for 3 hours.

The clones which appeared novel in sequence when originally compared with the nucleotide and protein databases have been recently re-entered. No additional matches with any of these sequences were found. It would appear that the clones with undetermined sequence homology may represent novel genes expressed at specific stages in the mammary gland. Although these clones contained putative open reading frames, no signal could be detected by Northern Hybridisation or RNAse protection Assay for each of these clones therefore it remains to be shown whether they do indeed represent cDNAs of very low abundnace.

3.4 Discussion

The results presented in this chapter demonstrate the optimisation of the technique of differential display for analysis in a mammary epithelial cell line and also with mammary gland tissue. The initial studies performed using HC11 cells confirmed that this technique was capable of identifying differentially expressed genes. β-casein, a milk protein known to expressed following prolactin treatment in an *in vitro* mammary gland model, was identified in the appropriate prolactin treated sample. *In vivo*, β-casein was detected at high levels in the mammary gland from day 10 gestation onwards. This expression profile mirrors that documented for β-casein during pregnancy (Ball *et al.*, 1988). These results validate the use of this technique to detect differentially expressed genes. β-casein is a highly abundant gene in differentiated mammary epithelial cells. The detection of genes in the HC11 cells (clone 44.1), and in mammary gland (clones 102.7, 124.1, 124.3 and 124.5) whose expression profile cannot be confirmed by conventional Northern Hybridisation and/or RPA suggests that differential display has the ability to amplify genes of low abundance in the cell. The advance from cell lines to using a whole organ system for differential display has allowed the isolation of genes that show more subtle changes in expression during pregnancy. For example clone 72.3, which gradually increases in expression as gestation progresses, may not have been isolated

if only one or two samples were being compared side by side. When analysing two samples side by side there is a tendency to overlook changes such as this in preference for genes which are switched on or off in one of the samples. The use of a time course of mammary development has allowed the detection of more subtle quantitative changes in gene expression, which themselves may be just as important to mammary gland differentiation as genes which either switch on or off in response to differentiation.

The description of differential display in 1992 has taken the analysis of cDNA expression one step forward. Until then the only techniques available for identifying differentially expressed genes were differential screening and subtractive hybridisation, both of which have limitations. These techniques are both time consuming, cumbersome, and show high levels of false positive results. Differential screening involves screening an appropriate cDNA library, plated in duplicate with radiolabelled single stranded cDNA from two different populations of cells (Watson and Demmer, 1994). Most of the plaques will hybridise equally to both probes, but some will show a difference in signal strength. These clones represent differentially expressed cDNAs. The major problems associated with this technique are that successive rounds of screening are required to eliminate false positives. Plaques are required to be screened at low densities to differentiate between signals therefore only a small number of clones can be screened at one time. The major drawback of this technique is that rare RNAs are poorly represented in the cDNA probe resulting in weak hybridisation signals. As a consequence there are difficulties in observing any differences between replica filters, and a problem in distinguishing these weak signals from background hybridisation levels. cDNAs of very low abundance (<0.1%) were found to be very difficult to clone using differential screening and subtractive hybridisation was originally thought to be the preferred technique for this group of low abundance cDNAs. Subtractive hybridisation is a more complex procedure to perform. It is based on producing single stranded cDNA from a treated population of cells. This cDNA is hybridised to purified mRNA from a related cell population e.g. untreated cells, and the resulting single stranded unhybridised cDNA purified from the hybridised molecules (Watson and Margulies, 1993). These should

represent cDNAs that are more abundant in one population of cells. Again this technique has its limitations, the major one being that it is biased towards abundant transcripts. Modifications in this technique have been developed and these involve biotinylating common sequences and removing them using streptavidin prior to hybridisation. As the technique is based on hybridisation, the efficiency of this step dictates the number of false positives generated e.g. where the hybridisation is poor a high proportion of clones will be isolated that are common to both cell populations. A common problem with both of these techniques is the inability to isolate very rare cDNAs from these screens. Differential display appears to have overcome the vast majority of the problems associated with the existing cDNA screening methods. As it is PCR based, it has the ability to identify and amplify transcripts of low abundance. Analysis using this technique was not biased towards transcripts that were of higher abundance. In this study we identified the milk protein β-casein, which itself represents around 15-20% of the mRNA present in the cell by late gestation. This is one of many milk proteins expressed within the mammary gland, but was the only one that was identified in this study. β-casein was identified using only one of the four available oligo dT primers and one of the thirty random 10bp primers. In a situation whereby gene expression was to be analysed then these primer pairs could be avoided and the problem overcome. Differential display affords a major advantage over the existing techniques because it is not time consuming to perform. From the stage of having the population of RNA prepared ready for use, to excising bands from the gel can take as little as 3 days. This allows rapid screening of a high proportion of cDNAs within a relatively short time scale. The prime advantage offered by using differential display to screen cDNAs is that a time course of samples can be compared side by side as opposed to the earlier techniques which were limited to comparing two populations. This allowed the analysis of quantitative changes in gene expression and also decreases the chances of producing false positive cDNAs.

Many modifications of the differential display technique have been described recently. These include modifications to the oligo dT primers e.g. the use of three one base anchored oligo dT primers which reduces the number of reverse

transcription reactions needed for each RNA sample and minimises the redundancy and under-representation of certain RNA species due to degeneracy of the primers (Liang *et al.*, 1994). Zhao and co-workers (1995) in addition to Diachenko and colleagues (1996) described the use of longer random primers (25-29mers). These longer primers allowed the use of optimal nucleotide concentration and a higher stringency PCR. Thermostable Taq DNA Polymerase has allowed longer PCR products which were said to result in more reproducible differentially expressed bands. These adaptations were in part combining the techniques of differential display and RNA fingerprinting. Lohmann and colleagues (1994) chose to perform display reactions on horizontal polyacrylamide gels and visualised by silver staining, which allowed them to overcome problems associated with excising DNA from a dried polyacrylamide gel. This also resulted in the removal of radiolabelled nucleotides from the display reactions. Chen and Peck (1996) took this adaptation one step further by transferring the PCR products onto a nylon membrane and staining with dioxygenin to detect cDNA bands. In contrast to this other researchers had sensitivity problems and thus switched from using ^{35}S radiolabelled nucleotide to ^{32}P (Trentmann *et al.*, 1995) or to ^{33}P (Tokuyama and Takeda, 1995). Other modifications to the technoque involved adapting primers in such a way as a facilitate use in a screening protocol e.g. Yeatman and Mao (1995) devised a method whereby PCR products could be used directly in an ribonuclease protection assay as well as for use in automated sequencing. In general, the majority of these refinements were subtle changes in the original strategy designed to overcome problems that individual groups had encountered with the technique. The only modifications we chose to perform from the original technique described in 1992 were the use of four oligo dT primers containing one fixed and one degenerate base at the 5'end as opposed to twelve individual oligo dT primers containing two fixed 5' bases, and the use of non-denaturing polyacrylamide gels. We did not attempt to make any further modifications to the technique, as it appeared to be working optimally within the system we chose to analyse. Differential display has become a widely accepted and utilised cDNA screening technique in the last 5 years and it only requires examination of the literature to see how widely used this technique has become for the analysis of gene expression. The model systems within which

differential display has been used are described in Table 3.3.

The study of expression patterns of a gene within different tissues is normally performed by Northern Blot analysis or RPA, and these were the methods we chose to use for analysis of the clones isolated by differential display. However, these techniques are not adequate for the detection of a very low abundance message. Although RPA is approximately ten fold more sensitive than Northern Blot analysis, it still has some limitations in detection. By definition differential display results in the cloning of mRNAs of very low abundance (Liang and Pardee, 1992). Generally, the option at this stage would be the preparation of polyA$^+$ selected mRNA and then Northern Blot analysis. However this is not always an option, and is dependent on the availability of tissue. Indeed, one of the major advantages conferred by using differential display was the ability to perform the reactions with just 5μg of total RNA, with no need to purify and enrich for mRNA. Therefore the major proportion of the starting material for differential display is required for the screening reactions. In our display reactions we excised four bands which we could not detect by either of these methods. As the availability of both mammary glands and mice were limiting, we prepared an enriched mRNA population from mammary epithelial cells grown in culture, and screened this with the clones. These results were inconclusive, which does not automatically mean that these clones were artefacts. The banding patterns for each of these clones was repeated in differential display reactions generated using a different starting RNA population, which would suggest that the clones did represent coding RNAs. The inability to detect a message in mRNA enriched cells may be due to these transcripts not being expressed within the mammary epithelial cell population. The detection problem appears to be a major stumbling block with this technique and is not a problem distinct to this particular study. An adaptation of conventional RPA has been described to overcome this problem. The technique was described by a research group who encountered similar problems with detection of clones isolated from differential display. They had incorported PCR into the RPA reactions, using the original differential display primers to generate sense strand cDNAs, which could then be hybridised against *in vitro* transcribed cDNA clones (Woo *et al.*, 1995). The result was a more sensitive RPA. A semi-quantitative

System Used	Genes Identified	Reference
NIH-3T3 cells stimulated with FGF-1	Delayed-early gene related to aldose reductase	Donohue *et al.*, 1994
Aortic smooth muscle cells (Glucose Induction)	3 clones, one of which has homology to human elongation factor-2	Nishio *et al.*, 1994
NIH-3T3 cells induced with FGF-1	3 clones identified, phosphofructokinase, fatty acid synthase, and Ca^{2+} ATPase	Hsu *et al.*, 1993
Osteoblast-like cells induced with vitamin D3	Stathmin	Kumar and Haugen, 1994
Squamous carcinoma cell line induced with ionising radiation	Elongation factor 1δ	Jung *et al.*, 1994
Mammary epithelial cells compared vs mammary carcinoma cells	Maspin, which has tumour suppressing activity	Zou *et al.*, 1994
Chronic cardiac rejection	5 genes upregulated in transplanted hearts, 2 of which are novel	Utans *et al.*, 1994
Mammary epithelial vs breast cancer cells	3 novel clones, expression pattern of only one confirmed	Liang *et al.*, 1992
Human epithelial ovarian cancer	3 novel genes	Mok *et al.*, 1994
p53 induced apoptosis	10 clones, only 3 known	Amson *et al.*, 1996
Thymocytes induced with dexamethasone	2 clones, one of which is novel	Chapman *et al.*, 1995
Preimplantation mouse embryo	4 clones, 3 novel	Zimmermann *et al.*, 1994

Table 3.3 Systems used for Differential Display Analysis. The table details the tissue examined and the cDNAs identified using this technique.

RT-PCR method may be used to confirm expression. Technically this is a very demanding technique and not suitable for screening a high number of clones, although it would appear to be more likely to detect expression than any of the preceding techniques. The problems with detection are not only limited to confirming expression. Identical problems may be encountered when obtaining full length cDNAs. The conventional method involves screening a cDNA library if an appropriate one is available. If the clone to be screened cannot be detected by the standard mRNA detection methods, it seems extremely unlikely that these cDNAs will be represented in the library at a level high enough to isolate full length clones. Another option would be to use a PCR based method for generating full length cDNAs, 5'→3' RACE (Rapid Amplification of cDNA Ends). This technique again in very demanding to perform and itself is wrought with technical difficulties. Overall, it would appear that differential display is the leading technique for the analysis of changes in gene expression. In particular it is demonstrated to amplify very rare cDNAs, which may not be detected by conventional mRNA screening methods. It is highly probably that further development of differential display will itself result in speeding up the development of novel screening techniques for the detection of very rare cDNA transcripts. This will allow the realisation of the full potential of this tool for analysis of gene expression.

3.5 Future Directions In The Analysis of Gene Expression

Since the start of the studies reported in this thesis a number of different approaches for the analysis of gene expression have been developed. These are described in the following section and advantages over the current techniques are discussed.

3.5.1 Serial Analysis of Gene Expression (SAGE)

Serial Analysis of Gene Expression (SAGE) allows quantitative and simultaneous analysis of a large number of transcripts (Velculescu *et al.*, 1995). The method is based on the production of cDNAs containing biotinylated linkers. These 3' cDNAs are subsequently digested with a restriction enzyme that recognises a 4bp sequence, and the resultant digested fragments are purified using streptavidin beads. The purified cDNA pool is then divided into two aliquots and each population is annealed to a linker; this linker contains a primer site and a restriction site for a class 2 restriction enzyme. Class 2 restriction enzymes cut 9bp downstream from their recognition sequence. This 9-13bp sequence tag contains sufficient information to uniquely identify the 3' end of a cDNA. It has been proven that a sequence as short as 9bp can distinguish approximately 260,000 transcripts given a random nucleotide distribution at the tag site. The second stage of this technique involves ligating the two cDNA populations and performing a PCR to amplify these products. This amplified reaction is then ligated to concatenate the 9-13bp sequence tags; this allows the efficient analysis of transcripts in a serial manner by sequencing multiple tags within a single clone. It has been estimated that approximately 60,000 tags required to be sequenced to make this technique powerful enough to give representative results. When two populations are compared side by side the ratio of tags between these two cell types are analysed and this leads to expression patterns deduced from the abundance of these individual tags. These 9-13bp tags of interest can then be screened against EST (expressed sequence tag) databases for any homolgoy and the EST can be obtained and used to generate a full length cDNA. In this manner SAGE can be used to examine expression profiles between normal and cancer cells (Zhang *et al.*, 1997), and may lead to identification of transcripts that are more abundant in cancer cells as compared to normal cells or vice versa. The major advantage this technique offers is the rapid analysis of thousands of transcripts within the cell in a relatively short time scale. This is obviously highly dependent on the rate of sequencing of the tags. Recently two papers have been published describing the use of SAGE to analyse changes in colorectal cancer (Zhang *et al.*, 1997) and to identify genes involved in p53-induced apoptosis (Polyak *et al.*, 1997).

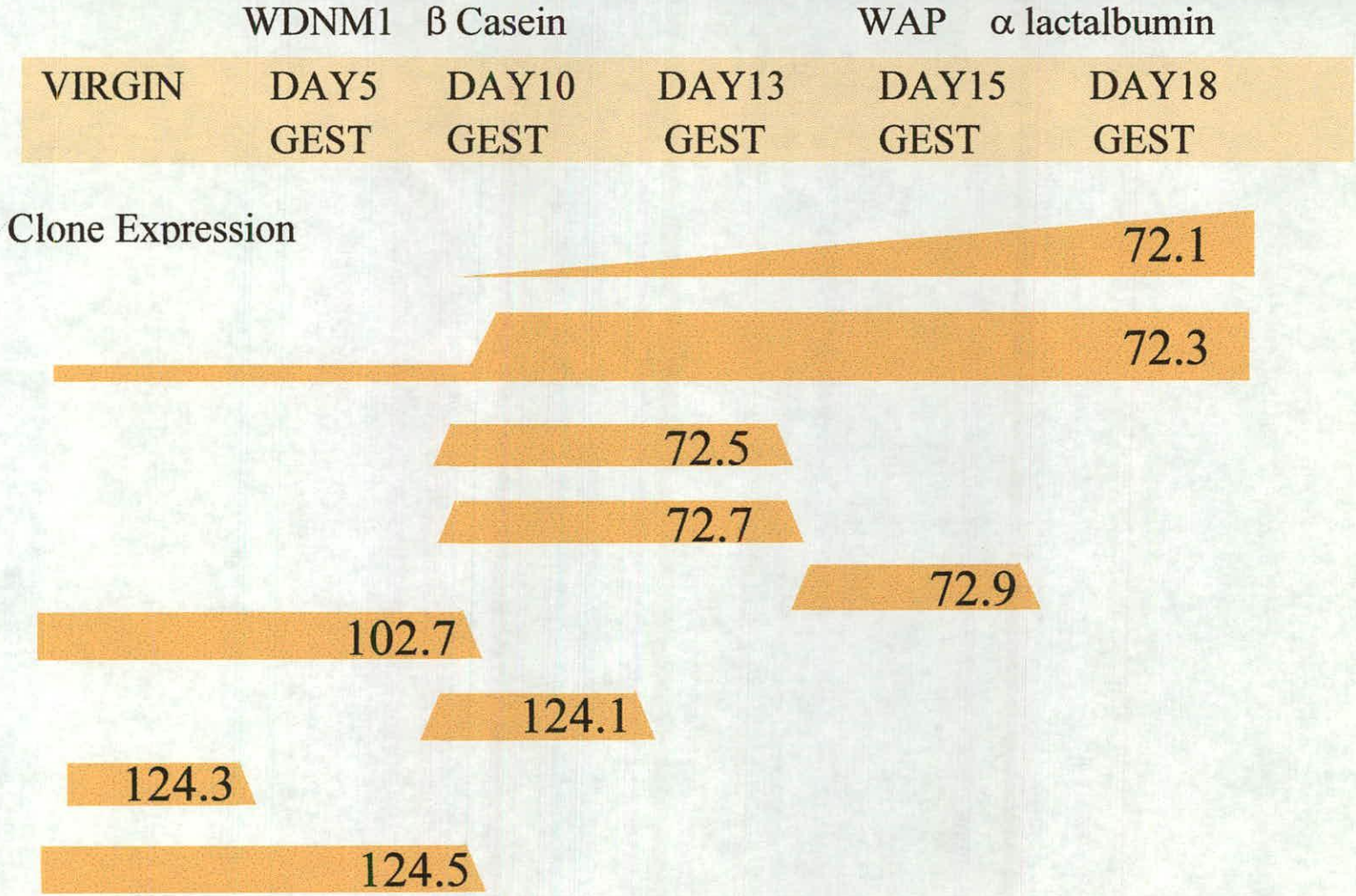
This technique appears to represent an advance in technology for the analysis of gene expression patterns. The major difference between the available techniques is that SAGE analysis quantitatively compares the expression profiles of a large number of transcripts within the cell. As SAGE is still a relatively new technique, it is difficult to determine whether it will replace differential display as a method for analysing gene expression changes. This clearly is dependent on whether SAGE is actually simple and rapid to perform, and on whether the technology to perform this analysis becomes commonly available.

3.5.2 Microarray Analysis

In the last few years a revolutionary new technology for measuring the expression of all genes in an organism has been described. The development of this technique was facilitated by technical advances which allow DNA fragments to be arrayed at high densities on a solid support for use in hybridisation experiments (reviewed in Johnson, 1998). The human genome mapping project has also provided the basis for this technique; it is estimated that all of the human genome will be sequenced within the next 5 years. This has resulted in a vast number of EST's being available to screen against. Two different methods for arraying DNA have been developed. In one, cDNA fragments generated by PCR are spotted onto poly-lysine coated glass slides. In the other, short (~25 base) oligonucleotides are synthesised on a glass surface. Both of these methods pack thousands of DNA fragments into a very small area: the current oligonucleotide chip displays all 6000 yeast genes on four 1.28 x 1.28cm chips; the DNA fragment microarrays fit the same information onto a single 1.8 x 1.8cm glass slide. The cDNA microarrays are generated by fixing unlabelled DNA fragments to the slide and probing with a complex mixture of labelled cDNAs or mRNAs. The labelled cDNAs represent the two populations to be compared and are labelled with different fluorochromes. The hybridised array is analysed using sophosticated microscopy and software that allows the quantitation of signal intensity. The major advantage of arrays over existing techniques is the significant increase in sensitivity. The small area occupied by the array results in

Figure 3.8 Summary of Differential Display Results. This diagram summarises the expression profiles of the clones examined in the mammary gland. WDNM1, β-casein, WAP, and α-lactalbumin are all milk proteins expressed at specific stages in gestation in the mammary gland. Clones 72.1 and 72.3 represent the cDNAs for β-casein and Tat Binding Protein1 respectively, whereas the remaining clones appear novel in sequence.

GENES EXPRESSED SPECIFIC TO STAGE



reducing the volume of the hybridisation solution and greatly increases the concentration of the probe. The use of a glass surface for hybridisation results in a smaller background hybridisation signal than the use of porous membranes. Both oligonucleotide and DNA microarrays permit very sensitive detection of gene expression: an mRNA present at a level of less than one molecule in 100,000 can be detected using this approach. An additional advantage of the microarray approach is that specific gene arrays can be prepared e.g. an array which encompasses all of the genes known to be expressed in the human mammary gland is available. The technology required to run this system is currently outwith the means of most laboratories, but commercial services are becoming available which may result in this technology becoming widely available. It is clear that microarray analysis represents a very powerful tool for the future of gene expression studies. The major advantage that both microarray and SAGE offer is the ability to screen thousands of EST's in one reaction. Should these techniques become widely used they will greatly enhance our knowledge of biological pathways.

The results described in this chapter demonstrate the use of differential display as a tool to identify changes of gene expression in the developing mammary gland. All of the genes identified in this study show changes at particular points in mammary development, which are key developmental junctures. This is demonstrated in Figure 3.8 where the expression profiles of the genes are compared to stages at which known genes become active. For example, clones 102.7, 124.1, 124.3 and 124.5 are all present in the early stages of mammary development when the gland undergoes a massive proliferation. Clones 72.5, and 72.7 are expressed at the stage when the mammary gland begins to differentiate (around day 10) as marked by the expression of β-casein. Clone 72.3 is expressed highly throughout the differentiation period in the gland, and the expression clone 72.9 peaks on day 15, the stage at which the mammary gland is thought to terminally differentiate as marked by the onset of expression of WAP. As these clones are all expressed at significant developmental stages in the mammary gland we suggest that these genes may represent key regulators of these process.

Chapter 4:

Analysis of TBP1 Expression in the Mammary Gland and Interaction of TBP1 with Related ATPases.

4.1 Introduction

In chapter 3 it was demonstrated that the expression profile of clone 72.3 (TBP1) increased ten fold by day 10 gestation, and that this was maintained until late gestation (day 18). As the majority of the remaining clones had no known sequence homology and in some cases the expression profiles could not be confirmed, it was decided to concentrate the remainder of this study on analysing the function of TBP1 in the developing mammary gland.

TBP1 was originally described as a protein that was capable of interacting with the HIV Tat transactivator and functioning as a transcriptional repressor. Nelbock and colleagues (1990) originally isolated TBP1 by screening a λgt11 fusion protein library prepared from Jurkat T cell cDNA with biotinylated Tat protein, and the TBP1 isolated in this manner was found to be predominantly nuclear in expression with highest expression in human cells. More recently TBP1 has been isolated from a variety of species e.g. rat (Rivkin *et al.*, 1997), *Plasmodium falciparum* (Hirtzlin *et al.*, 1994), *Dictyostelium discoideum* (Cao and Firtel, 1995), and yeast (Goyer, 1992). It is now widely accepted that TBP1 function is predominantly an ATPase activity associated with the ubiquitin dependent degradation pathway (described in section 1.6.1). TBP1 is one member of a family of ATPases, which have been assigned as members of the AAA family [ATPases Associated with a variety of cellular Activities] (Confalonieri and Duguet, 1995). The key feature of this family of proteins is a highly conserved module of 230 amino acids (the ATPases module) present in one or two copies in each protein. Despite extensive sequence conservation, the members of this family fulfil a very diverse range of cellular functions. These include cell cycle regulation, peroxisome assembly, 26S proteasome function, vesicle-mediated transport etc. It is hypothesised that these proteins may operate through two groups of distinct non-overlapping functional activities: (i) ATP-dependent proteasome function, and (ii) ATP-dependent anchorage of proteins. The six ATPases involved in proteasome function all contain a single ATPase module. As there had previously been some

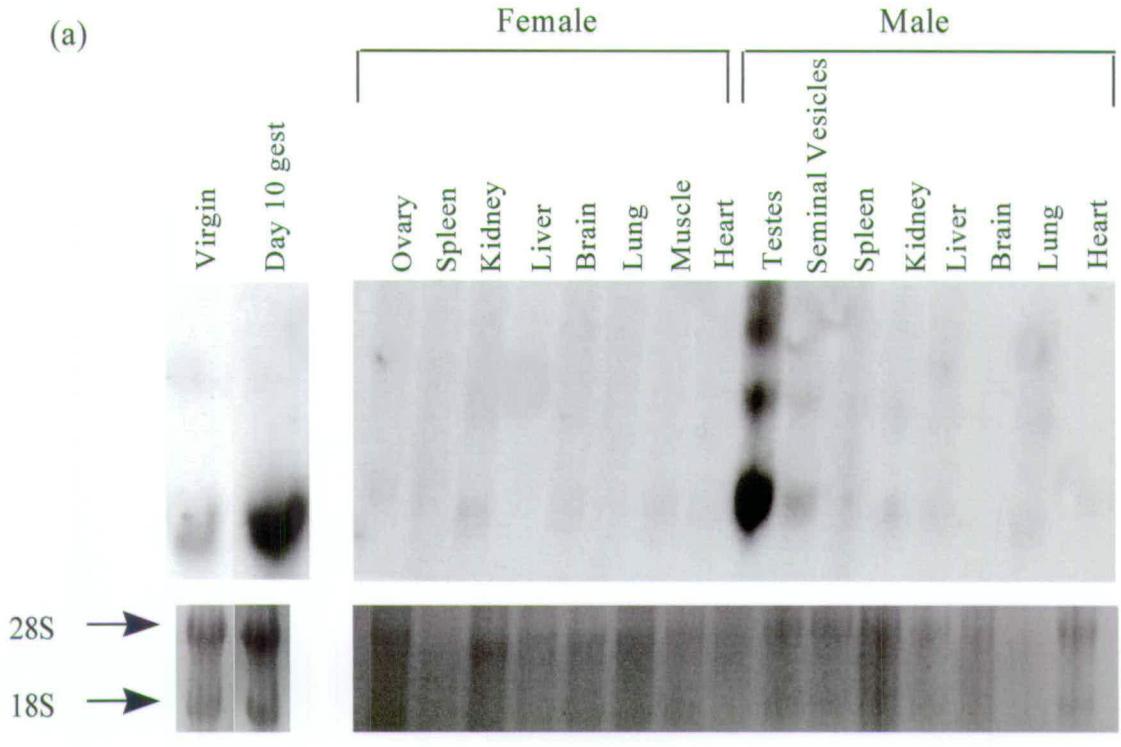
controversy over TBP1 function, the aim of the studies described in this chapter was to determine whether the expression profile examined was one for TBP1 alone or that of the 26S proteasome. We therefore examined the expression of 26S proteasome components in a mammary epithelial cell (MEC) line, KIM-2. KIM-2 cells were derived from the mammary gland of a mid-pregnant mouse expressing a temperature sensitive mutant of SV40 T antigen (tsA58) driven by the ovine β-lactoglobulin (BLG) milk protein gene promoter (Gordon, 1997). Mammary explant cultures were used to isolate stable cell lines that are now routinely used as a model system of mammary gland differentiation in our laboratory. KIM-2 cells functionally differentiate, as marked by the induction of the milk protein β-casein, in a similar manner to the existing mammary cell models. However, this cell line has retained the ability to express a late differentiation marker whey acidic protein (WAP) when cultured on plastic. No mammary model currently available will express WAP when cultured on plastic and KIM-2 cells have been used in this study as they provide a good *in vitro* model with which to study mammary epithelium development.

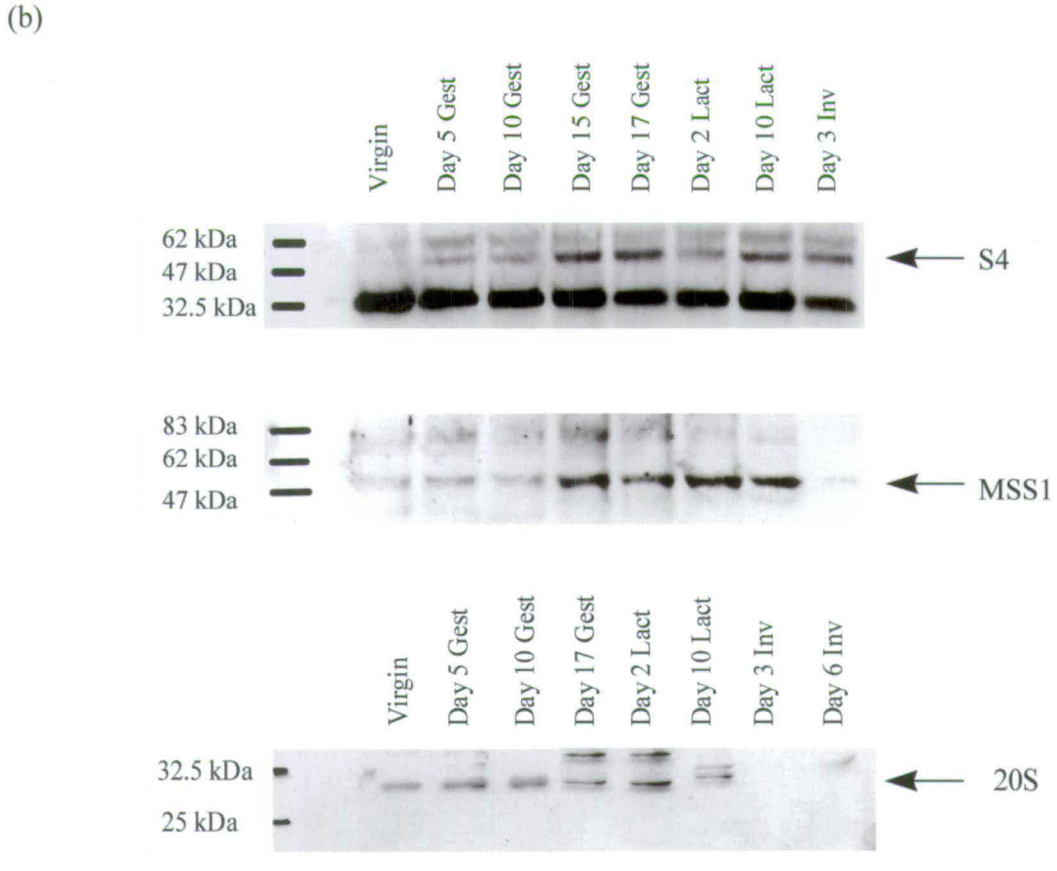
4.2 Upregulation of 26S Proteasome Components in the Developing Mammary Gland

The results generated by differential display analysis in chapter 3 documented a ten fold increase in expression of TBP1 at day 10 gestation, which was maintained until day 18 gestation. Examination of the expression of TBP1 in a wide range of other tissues showed that the level of TBP1 expression was low in the majority of other tissues tested, and this was comparable to the virgin mammary gland (Figure 4.1a). This observation suggested that the level of TBP1 was increased in mid to late gestation mammary gland relative to other tissues. The presence of TBP1 in the testes showed a relatively high level of expression with the presence of three transcripts. This result has been confirmed in the laboratory of Dr C Gordon (pers.

Figure 4.1 (a) Expression profile of TBP1 mRNA in a range of tissue samples. Total RNA was extracted from each of the tissues and 10μg electrophoresed, prior to blotting onto a nylon membrane. RNA was hybridised with radiolabelled clone 72.3, which represents the 3' 200bp of TBP1. The first panel represents TBP1 expression in the virgin and day 10 gestation mammary gland, followed by a range of RNA from either male or female organs.

(b) Expression profile of TBP1 Protein in the Developing Mammary Gland. Total cellular protein was extracted from mammary glands at different stages of gestation (Gest), lactation (Lact), or involution (Inv). 10μg protein was analysed by western blot using antibodies specific to 26S proteasome subunits, *S. pombe* S4 and 20S (kindly received from Dr. C. Gordon), and murine MSS1 (kindly received from Prof. J. Mayer). Although the antibodies used in this study were raised against specific ATPases subunits more than one band was commonly present on the developed blot. It was therefore assumed that the if a band was present at the correct size that this corresponded to the protein of interest. This may not have been the case for some of the antibodies used in this study therefore analysis of these results is limiting.


(a)
Female
Male
Virgin
Day 10 gest
Ovary
Spleen
Kidney
Liver
Brain
Lung
Muscle
Heart
Testes
Seminal Vesicles
Spleen
Kidney
Liver
Brain
Lung
Heart
28S
18S


(b)
Virgin
Day 5 Gest
Day 10 Gest
Day 15 Gest
Day 17 Gest
Day 2 Lact
Day 10 Lact
Day 3 Inv
62 kDa
47 kDa
32.5 kDa
S4
83 kDa
62 kDa
47 kDa
MSS1
Virgin
Day 5 Gest
Day 10 Gest
Day 17 Gest
Day 2 Lact
Day 10 Lact
Day 3 Inv
Day 6 Inv
32.5 kDa
25 kDa
20S

comm.) suggesting that the TBP1 expressed in the testes has alternatively spliced transcripts. The high levels of TBP1 in the testes may simply be due to a high basal rate of transcription that is often observed in the testes. A tissue like testes with a high rate of transcription may require elevated levels of degradation machinery to remove short lived proteins e.g. transcription factors which require a fast turnover.

The ATPases associated with proteasome function are believed to be predominantly involved in protein degradation, but other functions have been described for these proteins. In the main these tend to be assigned to a transcriptional function. To determine if the expression profile of TBP1 identified in the developing mammary gland was related to its function as a component of the ubiquitin-dependent protein degradation pathway, we examined the expression of related ATPases and of the 20S core of the proteasome in the mammary gland. Western blot analysis showed a corresponding increase in the expression of the ATPases MSS1 and S4 (Figure 4.1b). In contrast to this the level of the 20S proteasome α subunits appeared to be evenly expressed through gestation and early lactation. A decrease in the 20S proteasome was observed early in involution (Figure 4.1b). These results show that the high expression of these regulatory components of the 26S proteasome are maintained throughout lactation, and would suggest that the increase in TBP1 expression that was originally observed by differential display corresponds to an overall increase in the 26S proteasome. The antibody analysis on these ATPase subunits is limited due to the cross-reactivity of these antibodies with non-specific proteins.

4.3 Induction of TBP1 Expression During Mammary Gland Development is not Lactogenic Hormone Dependent

The expression profile observed by both differential display and Northern Blot analysis showed an increase in TBP1 expression around day 10 gestation. This is the stage when the mammary gland begins to express the milk protein β-casein for the first time, signifying an early differentiation event. To address the question as to

whether the increase observed in TBP1 expression was dependent on differentiation and lactogenic hormones, we switched to using an *in vitro* mammary gland model. The MEC line KIM-2 cells were chosen for this analysis as these cells can be induced to differentiate with the addition of the lactogenic hormones, prolactin and dexamethasone to their growth media. Figure 4.2a shows the morphological appearance of KIM-2 cells that have been induced to differentiate. After two days in culture these cells express the milk protein β-casein, and by four to six days they begin to express a second milk protein, WAP. This cell line was chosen for use over the HC11 cells that had been used for differential display analysis because KIM-2 cells have the ability to express WAP and are thus the most relevant *in vitro* model available for examining mammary gland development. Unfortunately, these cells were not fully characterised at the start of this study otherwise they would have been used for the differential display analysis. KIM-2 cells were induced to differentiate over a twelve day period and samples were collected for RNA analysis. Hybridisation of these samples with TBP1 revealed no induction in expression of TBP1 with differentiation of KIM-2 cells (Figure 4.2b). Protein levels of the core 20S proteasome were also examined in KIM-2 cells and a similar result was obtained (Figure 4.2c). No changes in the level of the core 20S proteasome were observed regardless of the differentiation status of the cells. These results suggested that the increase in TBP1 expression observed in the developing mammary gland was not occurring as a response to lactogenic hormones and also that the core of the proteasome is not hormonally regulated. Figure 4.2c also shows β-casein protein expression in these samples to confirm that the cells were functionally differentiated. Taken together these results suggest that there is an increase in the 26S proteasome as the mammary gland begins its differentiation process, and that this increase is not dependent on stimulation with lactogenic hormone. We have shown that MECs express TBP1, but do not act to upregulate its expression. A specific antibody against TBP1 would allow determination of which cells in the mammary gland express TBP1. This may enlighten our knowledge as to the function of an upregulation of TBP1 and the 26S proteasome in mammary gland development.

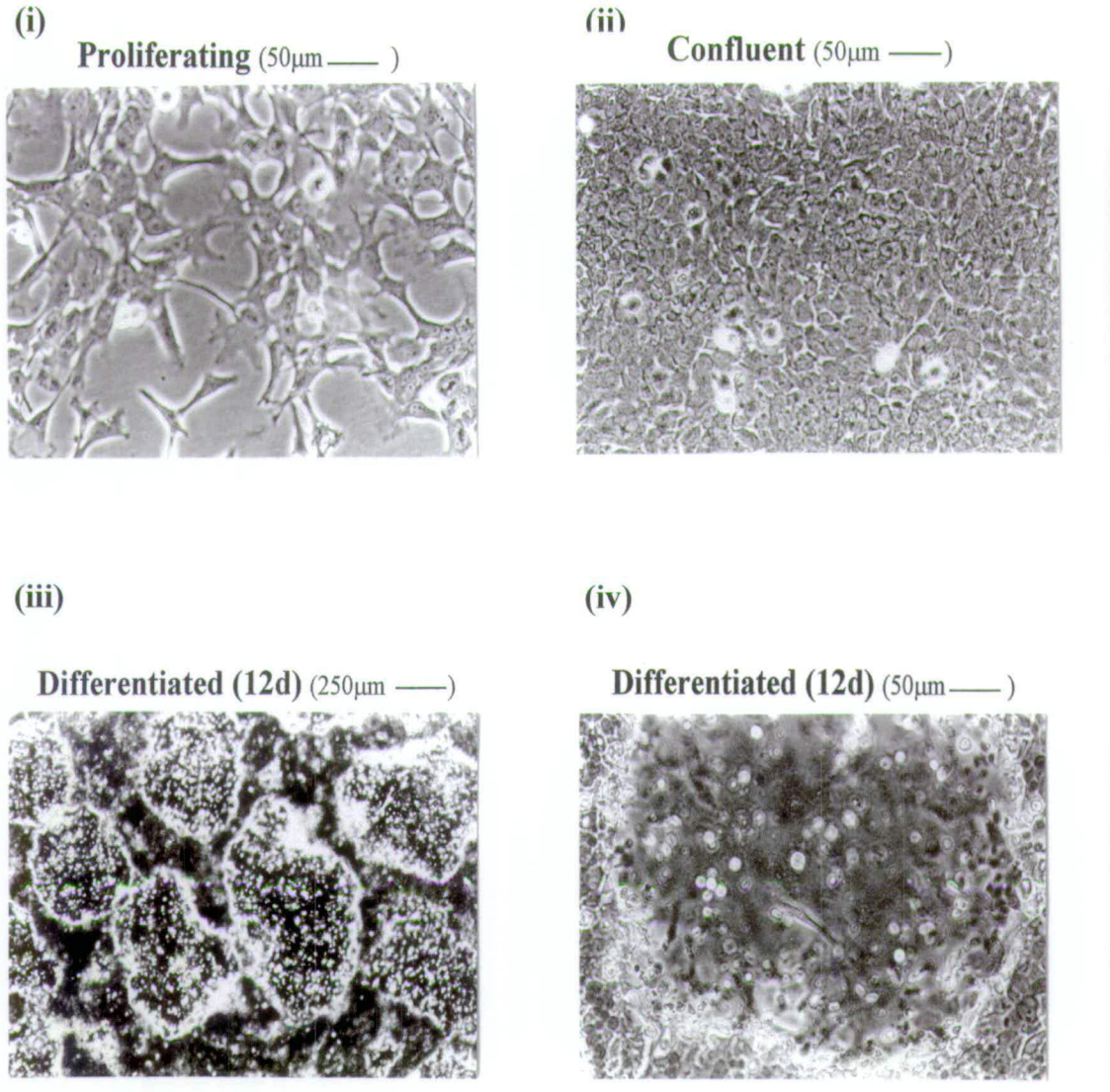
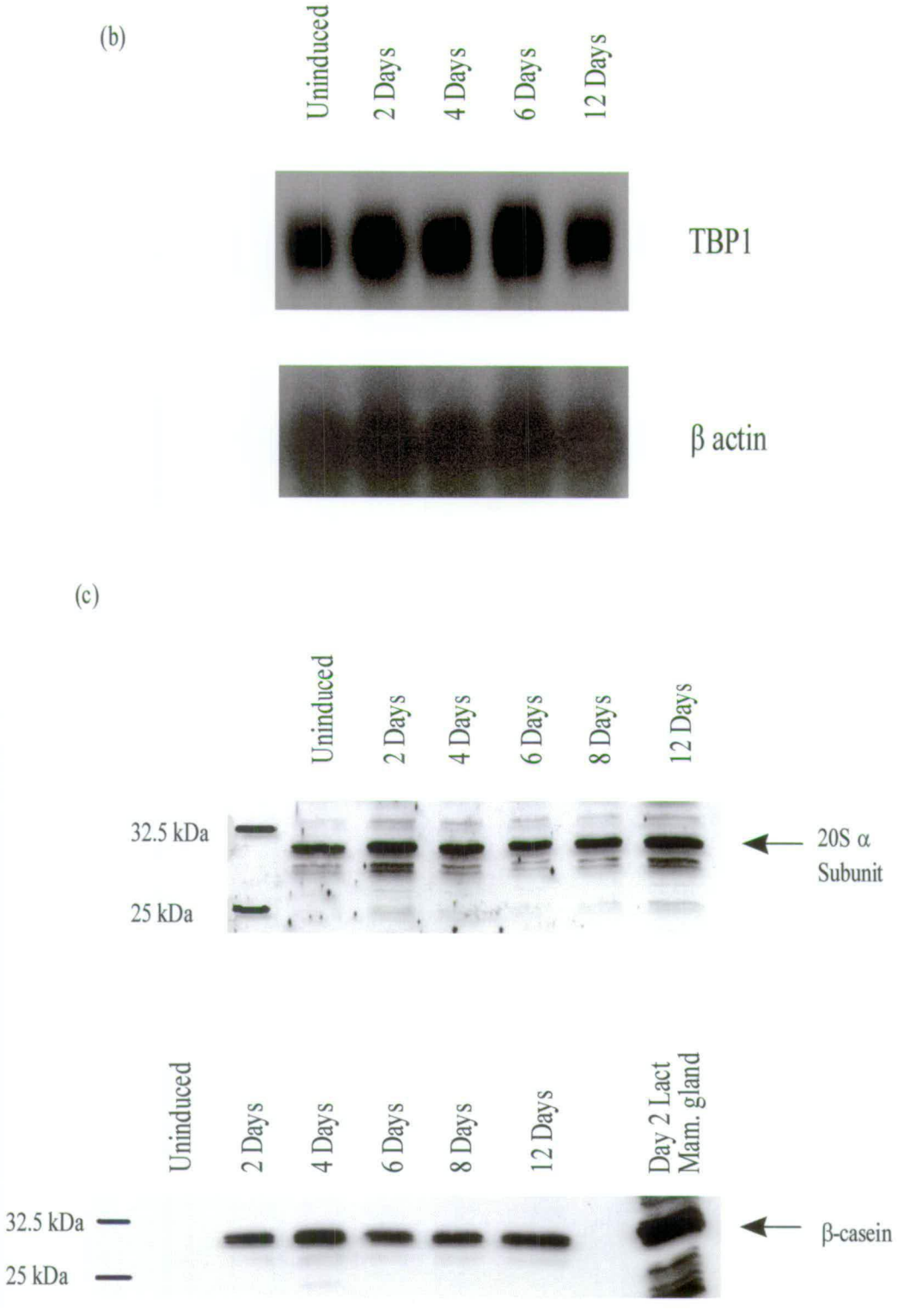


Figure 4.2a Morphological Appearance of KIM-2 Mammary Epithelial Cells. (i) Phase contrast of proliferating KIM-2 cells grown in maintenance media (MM) containing insulin and EGF. (ii) Phase contrast of confluent KIM-2 cells grown in MM, where a typical epithelial cobblestone morphology can be observed. (iii) Phase contrast of KIM-2 cells that had been differentiated for 12 days in the presence of the lactogenic hormones prolactin and dexamethasone. KIM-2 cells form domes or mammospheres when grown in the presence of lactogenic hormones on plastic. (iv) Higher resolution of differentiated KIM-2 cells. Myopeithelial cells can be observed on the surface of the mammospheres.

Figure 4.2 (b) Expression of TBP1 mRNA in KIM-2 Mammary Epithelial Cells. KIM-2 cells were grown to confluency and induced to differentiate over a 12 day period in the presence of dexamethasone and prolactin. Samples were harvested at various stages during this process and total RNA extracted from the cells. 10μg RNA was electorphoresed prior to Northern Hybridisation with a radiolabeled cDNA corresponding to the 3' 200bp of TBP1. The membrane was exposed to autoradiographic film overnight prior to stripping and probing with a cDNA corresponding to murine β-actin as a loading control.

(c) Expresison of the 20S Proteasome in KIM-2 Mammary Epithelial Cells. KIM-2 cells were induced to differentiate in the same manner as above. Samples were harvested and total cellular protein exptracted from these cells. 10μg protein was subjected to western blot analysis using a mixture of antibodies directed against the 20S proteasome subunits [*S. pombe* 20S antibody (from Dr. C. Gordon) and an ICN human 20S alpha subunit antibody]. The lower panel represents 10μg protein from the same cells subjected to western blotting using a murine β-casein antibody (kindly received from Dr. B. Binas).


(b)
Uninduced
2 Days
4 Days
6 Days
12 Days
TBP1
β actin
(c)
Uninduced
2 Days
4 Days
6 Days
8 Days
12 Days
32.5 kDa
25 kDa
20S α
Subunit
Uninduced
2 Days
4 Days
6 Days
8 Days
12 Days
Day 2 Lact
Mam. gland
32.5 kDa
25 kDa
β-casein

4.4 Screening of a Day 15 Gestation cDNA Mammary Gland Library For a Full Length TBP1 cDNA.

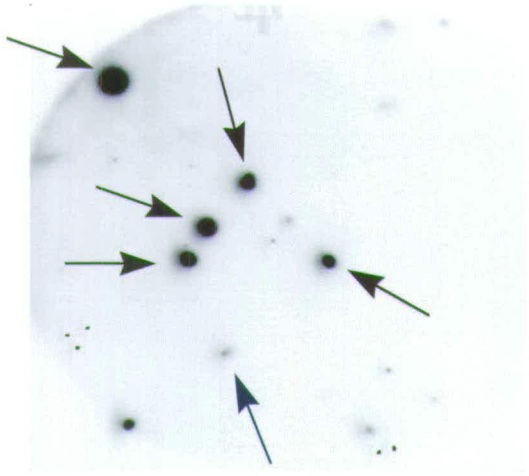
As the murine homologue of TBP1 had not previously been cloned, we chose to use the 3' 200bp cDNA fragment of TBP1 obtained by differential display to screen a day 15 gestation cDNA mammary gland library. The library had been prepared from a C57BL6/CBA strain of mouse by Dr C. Watson using a Stratagene Uni-Zap XR Kit. Following plating of the library, a mixed probe was used to screen the primary plaques. This was a mixture of radiolabelled cDNA fragments from clones 72.9 and 72.3 (TBP1). Hybridisation revealed two types of signal on the filters, a stronger and a weaker hybridisation signal (Figure 4.3a). Thirty six plaques were picked from the primary screen, containing a mixture of both types of signals. Approximately 50–100 pfu per plate were plated for the second screen and four lifts were taken from each plate as this screen was performed using both probes individually to differentiate between the signals observed. Hybridisation revealed a high number of positive plaques for TBP1 but no positive plaques for clone 72.9 (Figure 4.3b). Although the signals overlapped on duplicate filters at the stage of the primary screen and were weaker than the TBP1 signals, it appears that they were not related to clone 72.9. From the secondary screen with TBP1 26 plaques were obtained, which included 12 mixed and 14 single plaques. At this stage it was decided to excise all of the single plaques into pBluescript (described in section 2.9.4). Of the 14 clones excised, 13 of these gave colonies following excision. Rather than perform a tertiary screen, a plasmid DNA Southern Blot was performed as all of the clones excised represented single plaques. The blot was hybridised with the radiolabelled 3' TBP1 cDNA fragment used to screen the library originally. The results are shown in Figure 4.3c and show that of the 26 colonies screened, two contained no inserts and only one of all the clones contained an insert that did not hybridise to TBP1. Full length TBP1 clones of approximately 1.4kB were obtained from this screen as well as a range of smaller clones.

Figure 4.3 Screening of a Day 15 Gestation Mammary Gland cDNA Library for Full Length TBP1. (a) This panel represents the primary library screen, where plaques from a day 15 gestation mammary gland library (kindly provided by Dr. C. Watson) were screened with a radiolabelled probe containing a mixture of two cDNA fragments. The 3' 200bp of clones 72.3 (TBP1) and 72.9 were used to screen the library. Membranes were hybridised with this probe overnight prior to autoradiography for 3 hours. The arrows represent the clones picked for secondary screening. Two types of signal were detected, strong signals (black arrows) and weak signals (blue arrow).

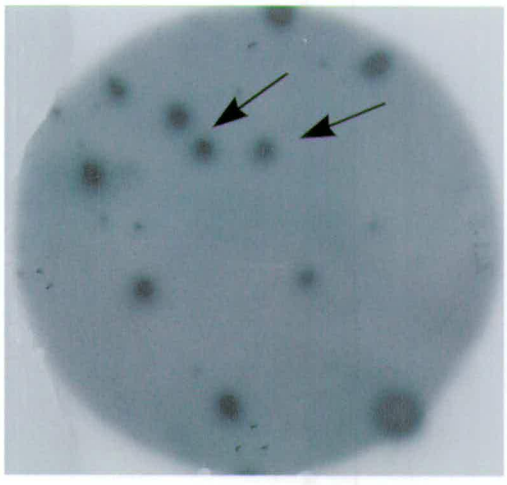
(b) Secondary Screen Using TBP1 cDNA Fragment. Positive plaques from the primary screen were re-plated prior to fixation on nylon membranes. Duplicate filters were prepared to screen with individual cDNAs on this screen. The example shown represents a membrane hybridised with the 3' 200bp of TBP1 and is a 3 hour exposure to autoradiographic film. The arrows represent the two plaques picked for subsequent analysis.

(c) Plasmid Southern Blot Using TBP1 cDNA Fragment. Positive plaques picked at the secondary screen were excised from the phagemid into the plasmid vector pBluescript. This DNA was transformed into competent cells and resultant colonies picked for analysis. Duplicate colonies were examined for each of the 13 plaques excised. Plasmid DNA was purified and 5μg was subjected to Southern Blotting onto nylon membrane. The membrane was hybridised using the 3' cDNA fragment of TBP1 generated by differential display and the membrane was exposed to autoradiographic film for 10 minutes.

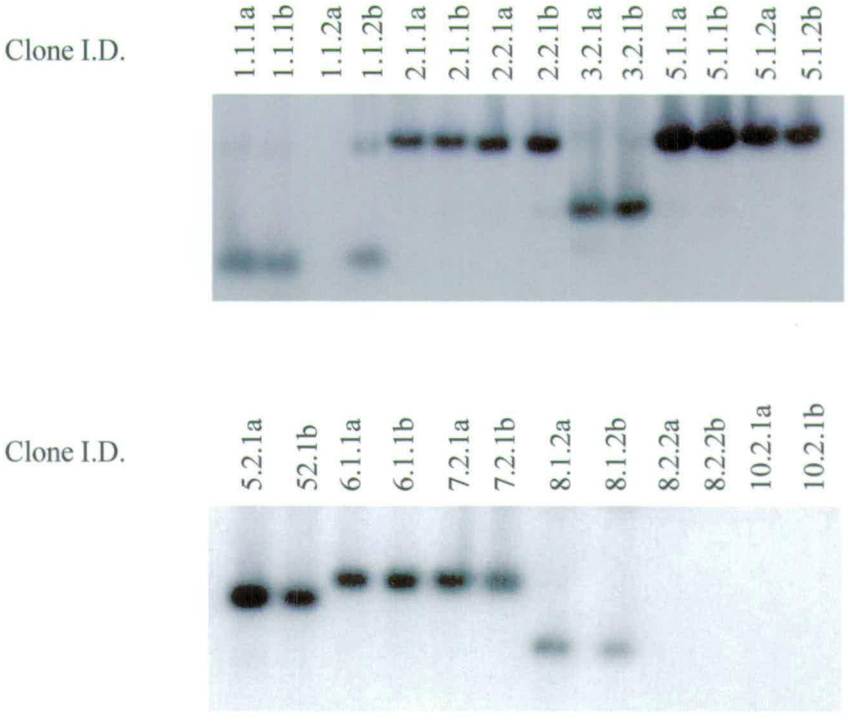
(a)



(b)



(c)


Clone I.D.
1.1.1a
1.1.1b
1.1.2a
1.1.2b
2.1.1a
2.1.1b
2.2.1a
2.2.1b
3.2.1a
3.2.1b
5.1.1a
5.1.1b
5.1.2a
5.1.2b
Clone I.D.
5.2.1a
52.1b
6.1.1a
6.1.1b
7.2.1a
7.2.1b
8.1.2a
8.1.2b
8.2.2a
8.2.2b
10.2.1a
10.2.1b

The library was plated out in duplicate for a second time to re-screen with clone 72.9. The possibility existed that no positive plaques were obtained for this particular clone as TBP1 appeared to be much more abundant in the library than clone 72.9. Thus it was decided that screening for this clone individually might allow the detection of plaques related to this cDNA which might previously been overlooked due to the intensity of signal for TBP1. In concert with this, clone 72.7 was screened against this library. Clone 72.9 peaked in expression on day 15 gestation and could be detected by Northern Blot analysis thus it was probable that this clone should be represented in a day 15 gestation cDNA library. Clone 72.7 peaked in expression on days 10 and 13 of gestation. Northern Blot analysis showed that this clone was expressed on day 15 gestation, albeit at lower levels than on days 10 and 13. Radiolabelled cDNA probes for both of these clones were screened against the library plaques and weak hybridisation signals were evident for both clones. Putative positive plaques were picked and plated out for a secondary screen. This second round screen showed that the plaques picked were not authentic positive signals, but background hybridisation. From this, it would appear that these cDNAs are not represented in this particular mammary gland cDNA library. This is assumed to be due to the low abundance of these particular clones.

Double stranded sequencing was performed on the cDNA fragments isolated from the library screen with TBP1. Figure 4.4a is a linear map showing some of the fragments obtained from the library and their position relative to full length TBP1. Fortunately, the fragments we had generated from the screening were overlapping in such a manner that they allowed TBP1 (1341bp) to be fully sequenced. Comparison of murine TBP1 to the nucleotide and proteins databases revealed 89% homology at the nucleotide level to human TBP1 and at the amino acid level this corresponded to 99% identity (Figure 4.4b). Only two amino acid changes were observed between murine TBP1 and all but the initial 26 amino acids of human TBP1. These were an isoleucine (I) to valine (V), and alanine (A) to arginine (R) change from human to mouse sequences respectively. The I to V change represents a conserved amino acid substitution. The murine TBP1 obtained in this study was 1341bp long, which translated to a protein of 404 amino acids. This was identical to the initial sequence

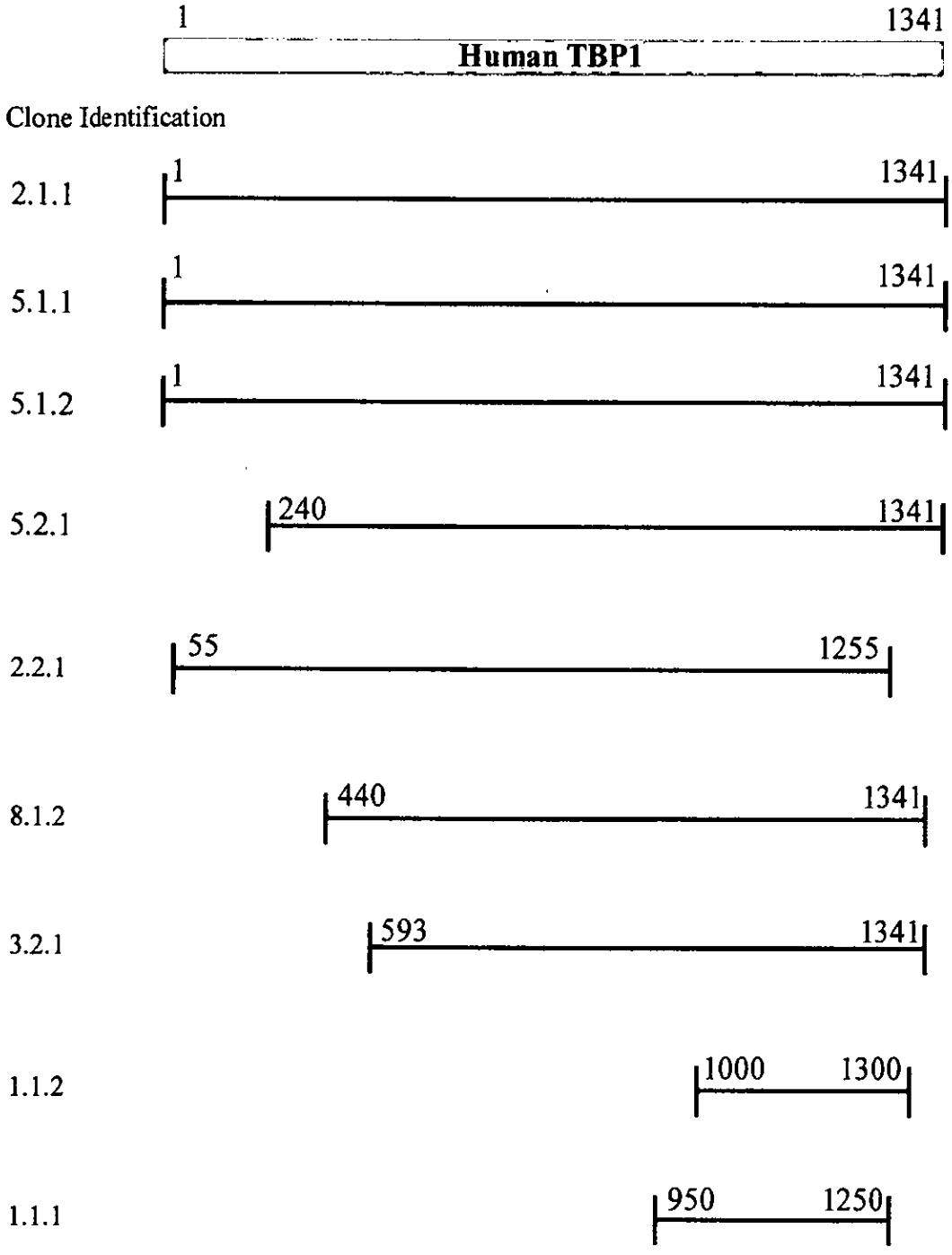


Figure 4.4a Linear Map of TBP1 Library Screen Fragments. These clones are represented as linear maps of the TBP1 fragments generated from screening a day 15 gestation mammary gland cDNA library. The clones detailed above were used to obtain the full length sequence of TBP1.

published for human TBP1 (Nelbock *et al.*, 1990). The same group later published a longer sequence for human TBP1 (Ohana *et al.*, 1993). Rescreening of the λgt11 fusion protein library resulted in the isolation of a 439 amino acid human TBP1. This sequence was seen to contain two ATG codons upstream of the initial proposed ATG start. The three full length murine clones obtained from the library screen were all 404 amino acids long with an ATG start at position 49. One ATG codon was found upstream but this was not in frame and would have only resulted in a protein of 21 amino acids. Only 10 of the remaining 18 codons 5' of the ATG start in the murine sequence matched with the human sequence. All of the mismatched codons were at the most 5' end of the murine TBP1 sequence. These results would suggest that the TBP1 expressed in the mammary gland corresponded to a protein of 404 amino acids. It would be of interest to examine the TBP1 form present in human breast tissue to determine if this truncation is a murine or a mammary tissue-specific event. It is possible that one of the other transcripts detected within the testes by Northern analysis may be identical to this longer form of TBP1 cloned by Ohana and colleagues (1993).

Murine TBP1 was 78% identical to the yeast TBP1 sequence suggesting that these proteins were very highly evolutionarily conserved. The high level of conservation in these ATPases is also seen in the components of the 20S proteasome. In eukaryotics, the 20S core is composed of seven different α and seven different β subunits. These subunits are very similar to those in *Thermoplasma acidophilum*, an archaebacterium that has only one α and one β subunit (Rubin and Finley, 1995). In *Thermoplasma*, ultrastructurally the 20S proteasome is almost indistinguishable from eukaryotic 20S particles. There is also sequence similarity between the α and β subunits families, suggesting that all subunits were derived from one single ancestral gene early in evolution (Peters, 1994).

Structurally the 6 ATPases associated with proteasome function all contain certain conserved sequence motifs. There are highlighted in Figure 4.4b and include a highly conserved functional ATPase module. Within this region there are 4 conserved sequences which are present in ATP- and GTP-binding proteins and ATP-

dependent DNA/RNA helicases. The nucleotide binding site or P-loop is a structure common to ATP- and GTP-binding proteins. The consensus sequence of the P-loop consists of a glycine rich sequence followed by a conserved lysine and a serine or threonine e.g. GXXXXGK[TS], where X denotes any nucleotide. In different protein families P-loops often have distinct features. For example in TBP1 this sequence is GPPGTGKT, which is also conserved within TBP7, MSS1, and Sug1 (Ohana *et al.*, 1993). The conserved lysine (K) residue was thought to be the most important residue in this motif as it confers the conformation of the P-loop and also directly interacts with the β- and γ- phosphates of the bound NTP (Saraste *et al.*, 1990). Two other regions show characteristics of GTP-binding proteins and the last region of homology highlighted has conservation with domain VI of a subfamily of DNA/RNA helicases. (Pause *et al.*, 1993). Another common feature of these proteins is a conserved carboxy-terminal cysteine residue (Cys^{361}). The function of this cysteine residue is unknown, but may have an as yet undefined catalytic function. The above conserved sites are all clustered within 100 amino acid residues from each other, and within the 6 ATPases they all begin around 250 amino acid residues from the amino terminal. The sequence conservation between the ATPases is markedly higher within the carboxy terminal of the proteins, whereas the structural homology is lowest at the amino terminal region (Lucero *et al.*, 1995). The N terminal amino acids of these proteins show the presence of a conserved leucine zipper-like structure, which may mediate protein-protein interactions. This structure is detailed in Figure 4.4c, and shows the heptad repeats within TBP1 and TBP7. Leucine is the most common amino acid at every seventh position, but methionine, isoleucine, and valine can appear as substitutes (Fu, 1992). There is a suggestion that this region of the protein may be involved in substrate recognition (Rechsteiner *et al.*, 1993).

Figure 4.4 (b) Comparison of Amino Acid Sequence of Human (H), Murine (M) and Yeast (Y) TBP1. Amino acid sequences were aligned using the Fasta program. The boxed region represents the highly conserved ATPase module. A highly conserved cysteine residue in represented by the yellow box. The blue boxed regions represent the putative nucleotide binding sites and the green box represents the helicase-like domain.

(c) Conserved Luecine-Zipper like Structure in TBP1. Comparison of human TBP1 and TBP7, and murine TBP1 amino-terminal regions. A highly conserved leucine-zipper like structure is found in these proteins. The hydrophobic amino acids forming the heptad repeat of the zipper are boxed, and the charged amino acids are depicted by "+" and "-" signs. Whilst leucine is the most common amino acid at even seventh position, methionine, isoleucine and valine are suitable alternatives.

(b)

```
MTBP1 M                                   STEEI  VQRTRLLDSE  IKIMKSEVLR
HTBP1 M                                   STEEI  IQRTRLLDSE  IKIMKSEVLR
YTBP1 MATLEELDAQ  TLPGDDELDQ  EILNLSTQEL  QTRAKLLDNE  IRIFRSELQR

MTBP1 VTHELQZMKD  KIKENSEKIK  VNKTLPYLVS  NVIELLDVDP  NDQEED
HBP1  VTHELQZMKD  KIKENSEKIK  VNKTLPYLVS  NVIELLDVDP  NDQEED
YTBP1 LSHENNVMLE  KIKDNKEKIK  NNRQLPYLVA  NVVEVMDMNE  IEDKENSEST

MTBP1   GANIDLDS  QRKGKCAVIK  TSTRQTYFLP  VIGLVDAEKL  KPNDLVGVNK
HTP1    GANIDLDS  QRKGKCAVIK  TSTRQTYFLP  VIGLVDAEKL  KPNDLVGVNK
YBP1  TQGGNVNLDN  TAVGKAAVVK  TSSRQTVFLP  MVGLVDPDKL  KPNDLVGVNK

MTBP1 DSYLILETLP  TEDYSRVKAM  EVDQRPTEQY  SDIGGLDKQI  QELVEAIVLP
HTBP1 DSYLILETLP  TEDYSRVKAM  EVDERPTEQY  SDIGGLDKQI  QELVEAIVLP
YTBP1 DSYLILDTLP  SEFYSRVKAM  EVDEKPTETY  SDVGGLDKQI  EELVEAIVLP

MTBP1 MNHKEKFENL  GIQPPKGVLM  YGPPGTGKTL  LARACAAQTK  ATFLKLAGPQ
HTBP1 MNHKEKFENL  GIQPPKGVLM  YGPPGTGKTL  LARACAAQTK  ATFLKLAGPQ
YTBP1 MKRADKFENL  GIRAPKGALM  YGPPGTGKTL  LARACAAQTN  ATFLKLAAPQ

MTBP1 LVQMFIGDGA  KLVRDAFALA  KEKAPSIIFI  [redacted]KA  FDSEKAGDRE
HTBP1 LVQMFIGDGA  KLVRDAFALA  KEKAPSIIFI  [redacted]KA  FDSEKAGDRE
YTBP1 LVQMYIGEGA  KLVRDAFALA  KEKAPTIIFI  [redacted]KA  FDSEKSGDRE

MTBP1 VQRTMLELLN  QLDGFQPNTQ  VKVIAATNRV  DILDPALLRS  GRLDRKIEFP
HTBP1 VQRTMLELLN  QLDGFQPNTQ  VKVIAATNRV  DILDPALLRS  GRLDRKIEFP
YTBP1 VQRTMLELLN  QLDGFSSDDR  VKVIAATNRV  DVLDPALLRS  GRLDRKIEFP

MTBP1 MPNEEARARI  MQIHSRKMNV  SPDVNYEELA  RCTDDFNGAQ  CKAVCVEAGM
HTBP1 MPNEEARARI  MQIHSRKMNV  SPDVNYEELA  RCTDDFNGAQ  CKAVCVEAGM
YTBP1 LPSEDSRAQI  LQIHSRKMTT  DDDINWQELA  RSTDEFNGAQ  LKAVTVEAGM

MTBP1 IALRRGATEL  THEDYMEGIL  EVQAKKKANL  QYYA
HTBP1 IALRRGATEL  THEDYMEGIL  EVQAKKKANL  QYYA
YTBP1 IALRNGQSSV  KHEDFVEGIS  EVQARKSKSV  SFYA
```

(c)

```
          36  - -   +   +   -   -   +   +   -    + 62
MTBP1     M S T E E T I Q R T R L L D S E I K I M K S E V L R V

          36  - -   +   +   -   -   +   +   -    + 62
HTBP1     M S T E E T I Q R T R L L D S E I K I M K S E V L R V

     40   +   + +       - -   -       - -   + - -   +  68
HTBP7 L Y S R Y K K L Q Q E L E F L E V Q E E Y L K D E Q K N L
```

4.5 Two Hybrid Analysis of Three ATPases of the 26S Proteasome

Biochemical studies have revealed that the 19S cap or regulatory complex is composed of 15 subunits, and that at least 6 of these proteins are thought to have an ATPase activity (Dubiel *et al.*, 1992). The assumption made from these studies was that an active 26S proteasome must contain all 6 ATPases. Studies from *Manduca sexta* have thrown confusion on this theory, as it appears that there may be a change in ATPase composition as the tobacco horn moth undergoes programmed cell death (Dawson *et al.*, 1995). An increase in the ATPases MSS1, TBP7 and S4 was detected, whilst TBP1 protein levels remained unchanged in the abdominal motor neurones and muscles during eclusion. The explanation for a change in proteasome subunit composition centred on a regulatory reprogramming which was thought to facilitate the degradation of newly ubiquitinated proteins. No other studies have reported a change in ATPase subunits with development. Despite this evidence, the major focus of proteasome research is based on the assumption that the 26S proteasome must contain all 6 ATPases for full function. Studies in yeast have shown that disruption of one of these ATPases, S4 causes a defect in mitosis (Gordon *et al.*, 1993). This phenotype was shown to be a consequence of the disruption of protein degradation as an increase in ubiquitinated proteins was observed. These results suggested that S4 was essential for proteasome function. The aim of the following work was to gain an insight into the organisation of the regulatory complex. To achieve this we cloned two additional murine ATPases, Sug2 and TBP7 by PCR and performed a yeast two-hybrid assay.

The study of protein interactions is of prime importance as these interactions play a critical role in the vast majority of biological processes. For example, the identification of interactions between viral oncoproteins and cellular tumour suppressor proteins, between components of signal transduction pathways, and between proteins involved in the regulation of the cell cycle have greatly increased the understanding of cellular functions. Two-hybrid analysis has been invaluable in studies to define regions of proteins that are responsible for specific interactions e.g.

SH2 and SH3 domains (Bartel and Fields, 1995). The yeast two-hybrid system is a genetic based assay for detecting protein interactions *in vivo*. It can be used to detect interactions between two known proteins or to screen libraries with a target protein, and was developed by Fields and Song in 1989. The assay exploits the observation that many transcriptional activators contain two independent domains, one containing a specific DNA binding activity and the other recruiting the transcriptional machinery (activation domain). The close proximity of these two domains is deemed sufficient to active transcription. The observation that several transcription factors have undetectable DNA binding activity and also that they were thought to act via protein-protein interactions led to the foundation of this technique. In the two-hybrid system, two fusion proteins are generated, one is a fusion of the DNA binding domain of a transcription factor and a test protein X. The other is a fusion between the test protein Y and an activation domain. Plasmids encoding these fusions are introduced together into a *Saccharomyces cerevisiae* strain that contains one or more reporter genes with upstream binding sites for the DNA binding domain present in the first hybrid. If the test proteins X and Y interact then the transcriptional activator is brought in close proximity to the DNA binding site and results in expression of the reporter gene (Figure 4.5a). The protein interactions successfully detected by this system include those found in a variety of subcellular compartments e.g. the nucleus, cytoplasm, and mitochondria. The most commonly used DNA binding domains are from the yeast transcription factor Gal4p and the *E. coli* repressor lexA. Gal4p and the herpes simplex virus VP16 protein are the preferred activation domains chosen for use with this system (Bartel and Fields, 1995).

The two-hybrid vectors used in this study are outlined in Figure 4.5b. These vectors, pAS2 and pACTII represent the DNA binding and activation domain vectors respectively. The recipient cells have different auxotrophic markers and the plasmids harbour the corresponding wild type genes e.g. leucine for pAS2 and trypotophan for pACTII. The pAS2 vector contains the coding region of the Gal4 amino-terminal region (residues 1-147) driven by a constitutive yeast alcohol dehydrogenase (ADH) promoter. The pACTII vector contains the coding region of

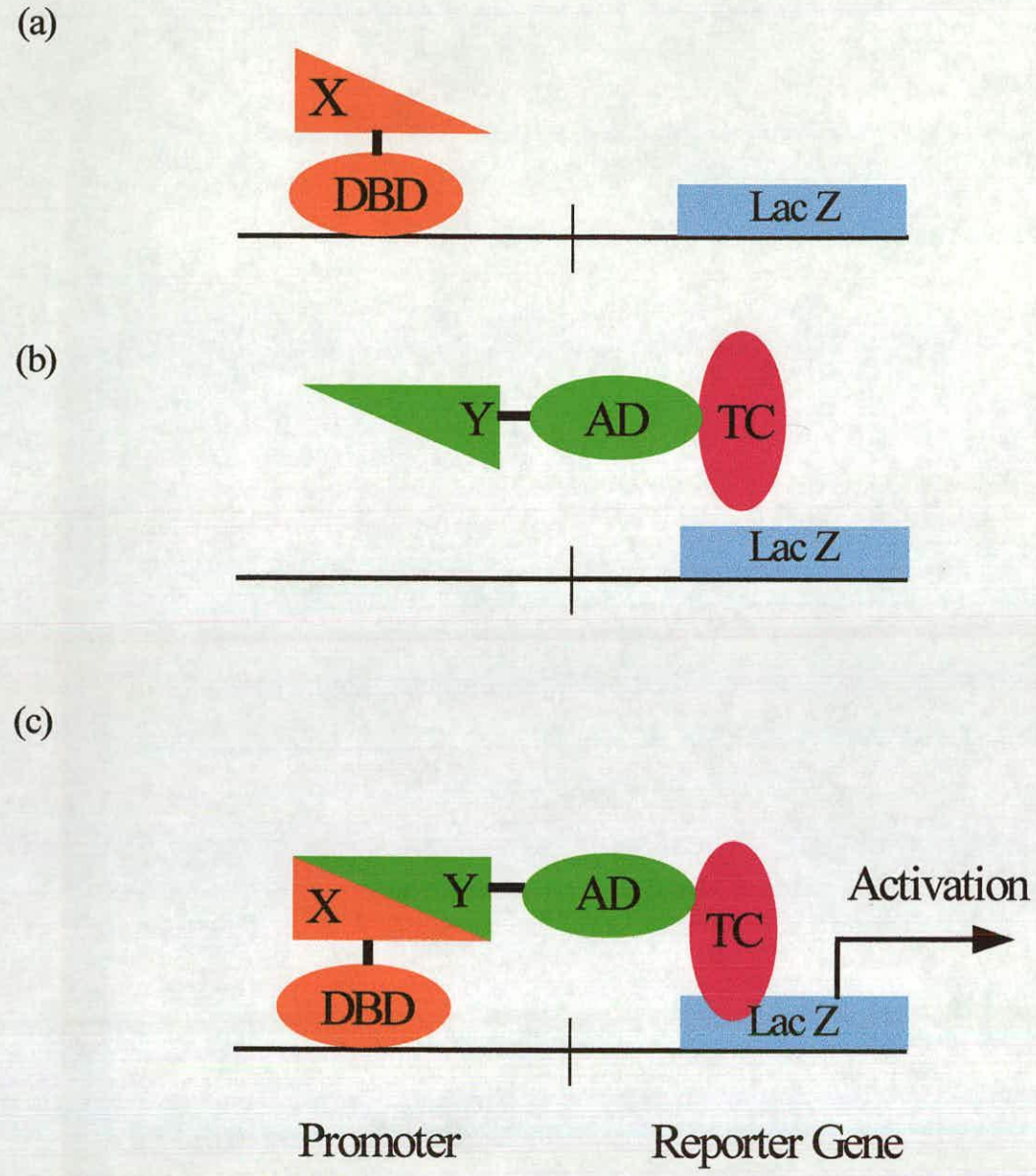
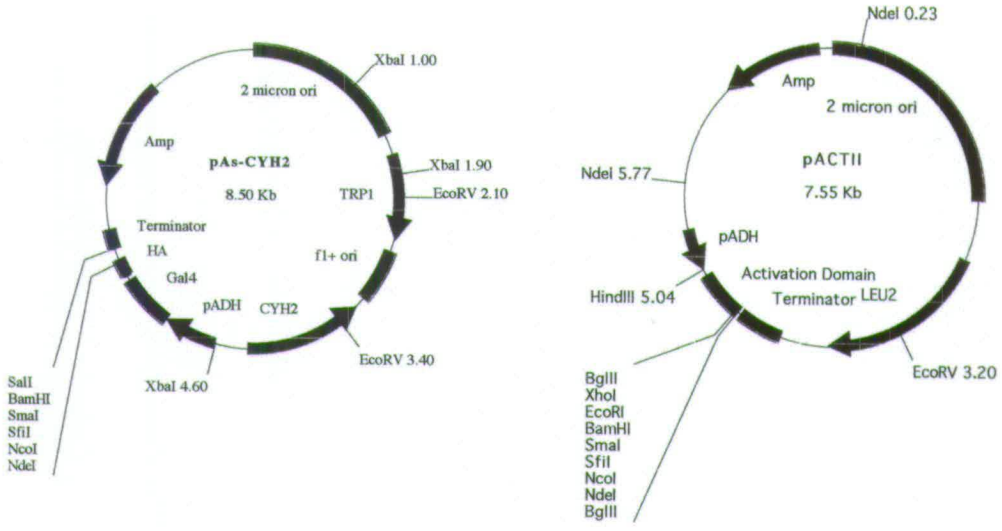


Figure 4.5a Basic Two-Hybrid System. (a) A hybrid consisting of a DNA binding domain (DBD) fused to protein X is unable to activate transcription of a reporter plasmid because it lacks an activation domain. (b) A hybrid consisting of an activation domain (AD) fused to protein Y fails to localise to the reporter gene. (c) If both hybrids are expressed in the same cell and protein X and Y interact, the activation domain and transcription complex (TC) are anchored to the DNA binding site and the reported gene is expressed.

the Gal4 carboxy-terminal region (residues 768-881) in addition to a nuclear localisation signal placed in frame with the Gal4 sequence. The vectors used in this study had a haemaglutinin (HA) epitope inserted between the Gal4 sequence and the multiple cloning site. This allowed detection of the expressed tagged fusion proteins. The reporter genes assayed in this test were HIS3, which allows growth selection and LacZ, whose activity was detected by a colourimetric assay. The fusion proteins to be analysed required cloning in frame with the Gal4 sequences and thus were generated by PCR amplification. As murine sequences for TBP7 and Sug2 were not available the human sequences were used to design primers to amplify the coding regions of these genes. Murine TBP1 was also amplified by PCR within the coding region to facilitate cloning. Restriction sites were incorporated into either end of the PCR products for cloning into the multiple cloning site. Each ATPase was cloned into both DNA binding domain and activation domain vectors to allow conformation of an interaction in both directions e.g. if TBP in pAS interacted with TBP7 in pACT, then TBP7 in pAS should also interact with TBP1 in pACT. In some cases, the ATPases had to be amplified twice as different restriction sites were required for the pAS2 and pACTII vectors. In general, only three primers were designed for each ATPase as the pAS vector contained a SalI restriction site that was placed on the 3' end of the amplified ATPase. This Sal I site when cleaved could be ligated to the corresponding XhoI site in the pACT vector. This resulted in only the 5' primer requiring different restriction sites for each vector and thus reducing the number of primers required. Figure 4.5c shows the resultant amplified PCR products. Sug2 was amplified from both human and murine cDNA because the murine homologue of Sug2 had not yet been identified and we were unsure how highly conserved the 5' sequence was around the ATG start site between the human and mouse protein. Initially the only Sug2 sequence available was that of *S. cerevisiae* and we were unable to generate murine Sug2 from the primers designed from yeast Sug2. Latterly the human sequence was published and this was used for more successful amplification. The PCR products were cloned into both a PCR cloning vector (TAII) and the two-hybrid vectors. The murine ATPase MSS1 cloned into pAS2 and pACTII was kindly received from Dr Gordon McGurk. The transformed colonies were picked and inserts were checked by restriction digestion of plasmid DNA.

(b)



(c)

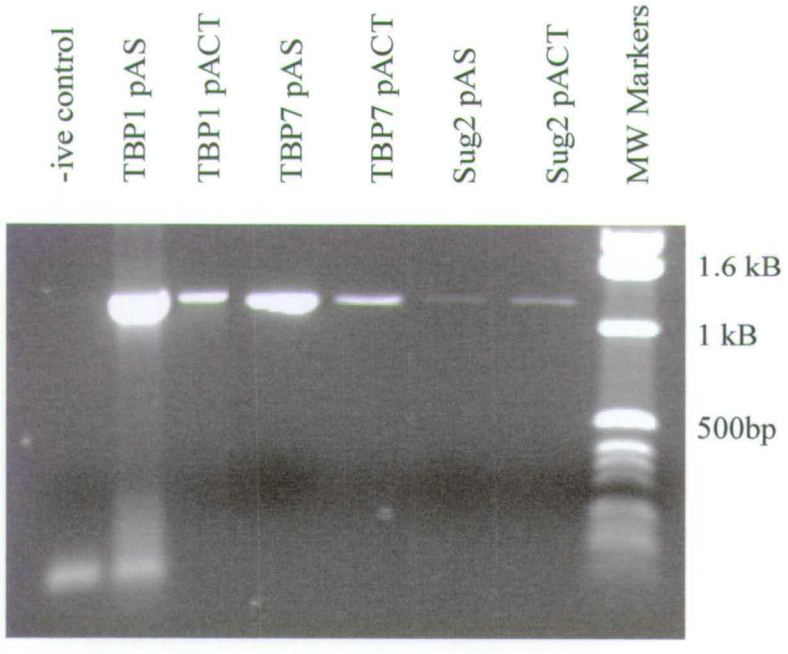


Figure 4.5 (b) *S. cerevisiae* two hybrid vectors. pAS-CYH2 contains the Gal4 DNA binding domain and pACTII contains the Gal4 Activation domain. PCR products were preferentially cloned into either 5' NdeI or NcoI sites and to the 3' SalI or XhoI restriction sites.

(c) Two Hybrid PCR products. PCR products were generated from day 15 gestation mammary gland cDNA using primers designed to generate 5' and 3' restriction sites on these ATPases. An aliquot of the reaction was run on a 1% agarose gel to check the reaction was successful. The -ive control is an amplification reaction performed in the absence of template cDNA.

(a) TBP7

```
1   MKQDEIPALSVSRPQTGLSFLGPEPEDLEDLYSRYKKLQQELEFLEVQEE
51  YIKDEQKNLKKEFLHAQEEVKRIQSIPLVIGQFLEAVDQNTAIVGSTTGS
101 NYYVRILSTIDRELLKPNASVALHKHSNALVDVLPPEADSSIMMLTSDQK
151 PDVMYADIGGMDIQKHEVREAVELPLTHFELYKQIGIDSPRGVLMYGPPG
201 CGKTMLAKAVAHHTTAAFIRVVGSEFVQKLPGEGAPGMSGMCSALLRRNA
251 PAIFFMDKLMPLPPRDSMPQTGADREVQRILLELLNQMDGFDQNVNVKVI
301 MATNRADTLDPALLRPGRLEGKIEFPLPDRRQKRLIFSTITSKMNLTEEV
351 DLEDYGARPDKISGADINSICQESGMLAVRENRYIVLAKDFEKAYKTVIK
401 KDEQE
```

(b) Sug2

```
1   MADPRDKALQDYRKKLLEHKEIDGRLKELREQLKELTKQYEKSENDLKAL
51  QSVGQIVGEVLKQLTEEKFIVKATNGPRYVVGCRRQLDKSKLKPGTRVAL
101 DMTTLTIMRYLPREVDPLVYNMSHEDPGNVSYSEIGGLSEQIRELREVIE
151 LPLTNPELFQRVGIIPPKGCLLYGPPGTGKTLLARAVASQLDCNFLKVVS
201 SSIVDKYIGESARLIREMFNYARDHQPCIIFMDEIDAIGGRRFSEGTSAD
251 REIQRTLMELLNQMDGFDTLHRVKMIMATNRPDTLDPALLRPGRLDRKIH
301 IDLPNEQARLDILKIHAGPITKHGEIDYEAIVKLSDGFNGADLRNVCTEA
351 GMFAIRADHDFVVQEDFMKAVRKVADSKKLESKLDYKPV
```

Figure 4.5d Sequence of Murine TBP7 and Sug2. Murine TBP7 and Sug2 coding cDNA sequences were amplified for use in the Two Hybrid Assay. Automated sequencing of these cDNAs were performed by Oswel DNA Services. The upper sequence is TBP7 and the lower sequence Sug2. Translation was performed using the Blast database (http:\\.www.ncbi.nlm.nih.gov) and sequence homologies were compared.

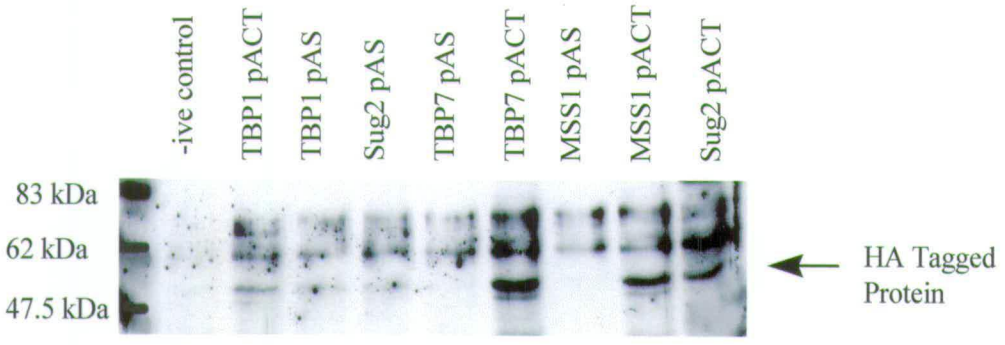
Each clone to be used in the two-hybrid analysis was sequenced to determine that no errors had been introduced during the PCR amplification. The amplification was performed in the presence of high fidelity Taq DNA polymerase, which is a proof-reading enzyme designed to reduce the number of errors introduced by PCR into a sequence. Figure 4.5d shows the amino acid sequences of murine TBP7 and Sug2. They show 87% and 85% identity to their human counterparts respectively.

Once PCR products were cloned into the appropriate vectors, they were subsequently transformed individually into the *S. cerevisiae* strain used for the interaction assay, Y190 cells. These clones were grown on synthetic complete (SC) plates containing all the nutrients required for growth e.g. adenine, histidine, and either leucine or tryptophan depending on the vector. The resultant colonies were picked and grown in liquid culture for 48 hours, and protein subsequently extracted. Western Blot analysis was performed using an HA antibody to detect tagged fusion proteins (Figure 4.6a). These results showed that all the fusion proteins were expressed although not at high levels in some cases. One striking observation from this analysis was that the constructs expressed in the activation domain vectors appeared to be expressed at a higher level than those in the DNA binding domain vectors. The observed difference was not due to any difference in the constructs because in the case of TBP7 the same PCR product was cloned into both pAS and pACT vectors. The pAS vector must contain some intrinsic property that results in lower expression of fusion proteins in comparison to identical constructs expressed in the pACT vector.

The yeast reporter strain used for the interaction assays was Y190, which had a leaky HIS3 reporter gene (Emilsson *et al.*, 1993). This required the addition of the competitive inhibitor 3-aminotriazole (3-AT) in the medium, which inhibits any basal HIS3 activity. The test proteins were simultaneously transformed into the reporter strain and plated out on selective media. Growth on 3-AT plates was analysed approximately 7 days later. As a result of using growth selection as one of the reporters, the growth observed on 3-AT plates, relating to his^+ colonies was slow. Colonies were re-isolated on selective plates, allowed to grow for a further 4-5 days

and then tested for β-galactosidase activity. If the his^+ phenotype observed was due to the restoration of Gal4 activity via protein interaction, then the LacZ reporter gene should be induced. In addition to testing the bait proteins against each other, they were also tested alone and with two unrelated proteins, SNF1 and SNF4, which were used as a positive control for interaction. SNF1 is a member of a protein kinase family and is essential for the transcription of glucose-repressed genes in response to glucose starvation, and SNF4 is the activating subunit of SNF1 (Jiang and Carlson, 1997). Results from the interaction assay showed that the transformation reaction was efficient as detected by the number of colonies on plates containing no selective pressure. On four separate occasions when the assays were performed, the results suggested that TBP1 in pAS could activate transcription alone (Figure 4.6b). Most of the results obtained were with TBP1 pAS interacting with other bait proteins. TBP1 in pAS was seen to drive LacZ expression on its own, in conjunction with an unrelated protein SNF4, with TBP1 pACT, with Sug2 pACT, with MSS1 pACT, and with TBP7 pACT. Since all of these results could be due to TBP1 pAS activating transcription on its own, it therefore cannot be assumed to represent an interaction. A weak interaction was observed between the ATPases Sug2 pAS and TBP1 pACT, but could only be observed in one direction and could not always be detected. It is occasionally the case with this assay that a real interaction can only be demonstrated in one direction. The reasoning for this is unclear but may be due to the hybrid protein not being stably expressed in yeast. It is also a possibility that if one protein is more highly expressed than another, as is the case for proteins expressed in the pACT vector, that this alter the dynamics of the interaction. This may result in a bias towards homodimerisation of the more abundant protein if its physiological interaction with the lesser expressed protein is normally of low affinity. The results presented in this thesis may provide evidence suggesting that some of these ATPases could play a transcriptional role in the cell. TBP1 in the presence of a DNA binding domain appeared to be able to activate transcription of a reporter gene either on its own or more strongly with unrelated proteins. The results generated for these ATPases by two-hybrid conflict with previous results that suggested TBP1 and TBP7 could heterodimerise (Ohana *et al.*, 1993). More recent biochemical studies have revealed further details on interacting proteins within the regulatory complex

(a)



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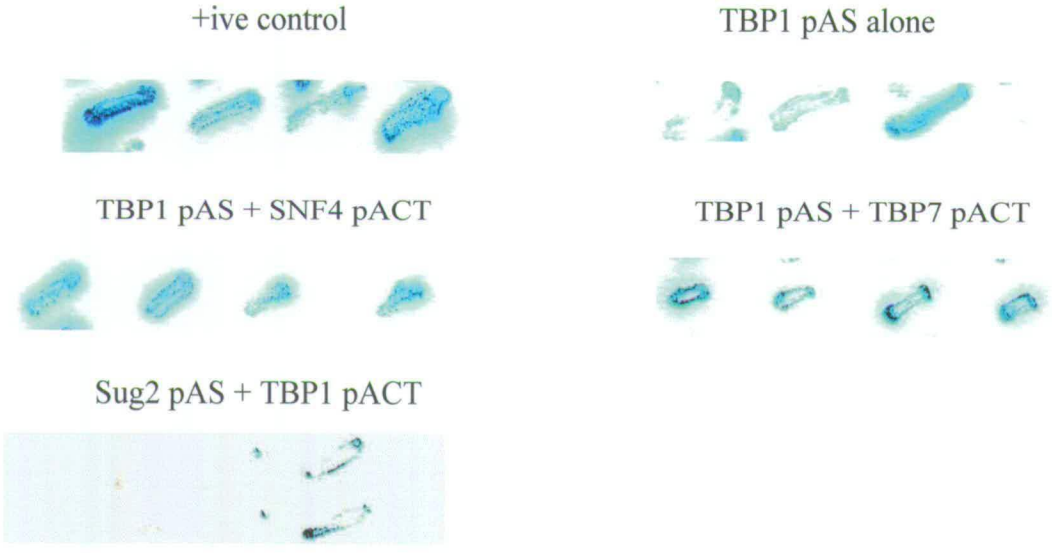


Figure 4.6 Two Hybrid Analysis. (a) Whole cell extracts were prepared from *S. cerevisiae* Y190 cells expressing the two hybrid bait proteins. 10 μg protein was subjected to SDS/PAGE analysis and subsequently blotted. Membranes were probed with a Haemaglutinin antibody (Sigma) and detected using ECL (Amersham). The –ive control is represented by Y190 cells alone. (b) Results of Two Hybrid Interactions Assays. In all cases 4 clones stained with Z Buffer have been shown, except Sug2 pAS + TBP1 pACT (where 8 clones are shown). The +ive control repesents an interaction between the yeast protein SNF1 and SNF4.

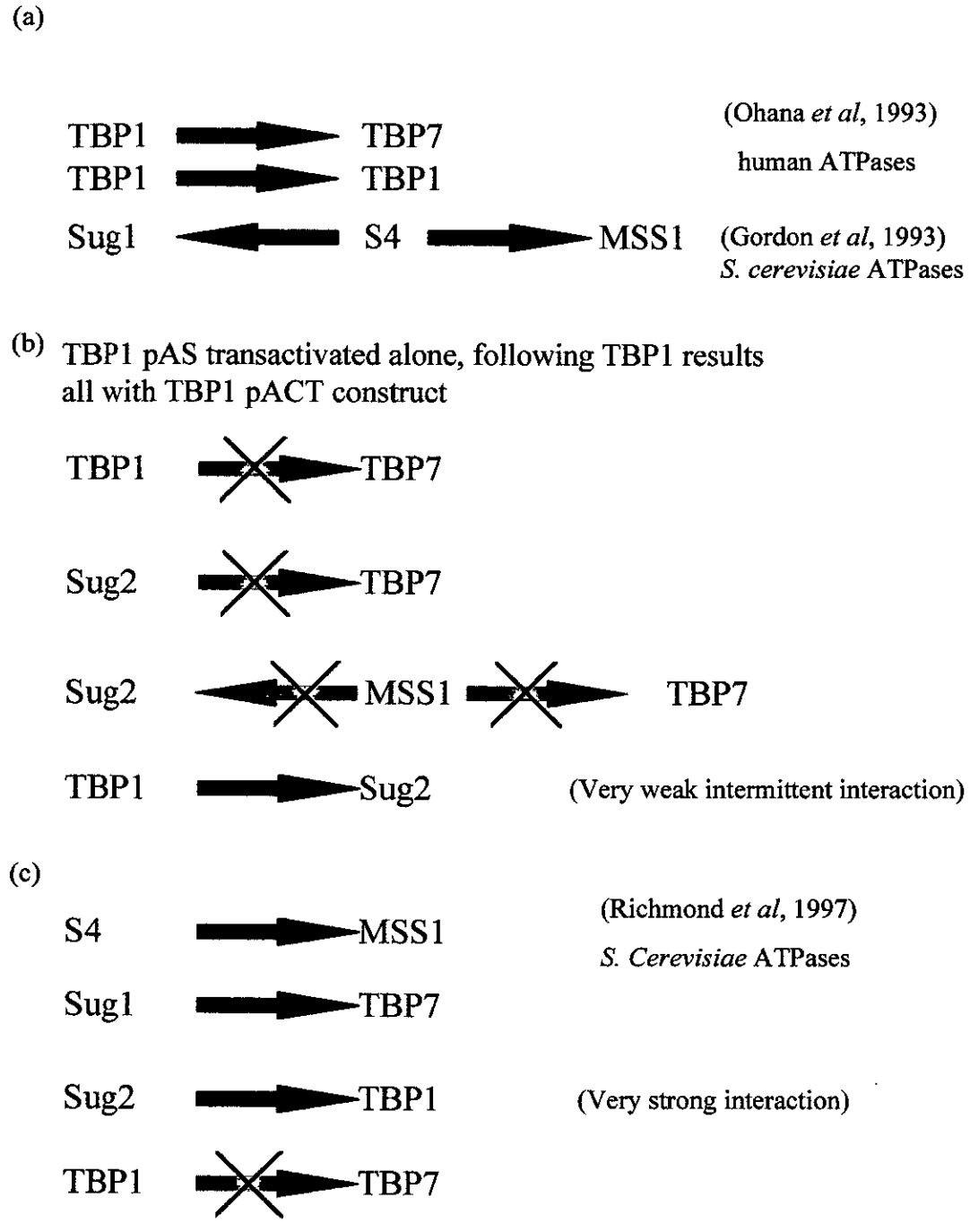


Figure 4.7a Summary of Two Hybrid Results. (a) Interactions that were known prior to the start of this study. Blue arrows represent positive interactions and grey arrows no detectable interaction. Both of the studies in this section were performed using the yeast 2 hybrid system. (b) Results generated in this thesis. TBP1 cloned into the DNA binding domain vector could activate transcription alone. The only other interaction which was detected was between TBP1 and Sug2 and this was a weak interaction which could not always be observed. (c) Nitrocellulose binding assay results determined that Sug2 and TBP1 interacted strongly and that the other 4 ATPases did not interact with either of these proteins.

(Richmond *et al.*, 1997). This group have shown by nitrocellulose binding assay that TBP1 and TBP7 do not interact, and that TBP1 and Sug2 strongly interact with one another but not with any other ATPases. Figure 4.7a summaries the results of the two hybrid analysis presented in this thesis and other available data on interactions between proteasome ATPases.

4.6 Discussion

The results presented in this chapter determined that the original increase in TBP1 expression that was observed by differential display was related to an overall increase in the 26S proteasome. Until recently it was unclear whether all 6 ATPases were actually a functional part of the 26S proteasome. This was due to a number of these ATPases being assigned functions relating to transcriptional control e.g. TBP1 was described as a transcriptional repressor (Nelbock *et al.*, 1990), and Sug1 as a transcriptional mediator (Fraser *et al.*, 1997, Weeda *et al.*, 1997, and Swaffield *et al.*, 1995). Biochemical purification of the proteasome complex revealed that 6 ATPases were part of the regulatory complex, and this led to the reclassification of these ATPases as components of the proteasome. The results in the mammary gland suggest that the increase in TBP1 expression observed was very likely related to TBP1 function within the 26S proteasome. The results also showed a similar increase in expression of two other ATPases, S4 and MSS1 although there was no change in the level of the core of the proteasome during this time. These results fit the predicted model for proteasome assembly; it is hypothesised that assembled 20S proteasomes sit within the cell and become functional when they are bound by a 19S regulatory complex or an 11S activator complex. At present there is some debate as to whether 20S proteasomes alone can degrade proteins; degradation by this complex has been observed *in vitro* but it is unclear whether this process occurs *in vivo*. These results suggest that there is no change in these particular ATPases as the mammary gland undergoes its differentiation process. Dawson and colleagues

(1995) observed changes in the ATPase composition of the proteasome as the tobacco horn moth underwent developmental changes. These results were interpreted to suggest that the proteasome could change its regulatory subunit composition dependent on the developmental process occurring. From the results presented in this chapter, this would appear not to be true for the mammary gland. Three ATPases were examined in the mammary gland during proliferation, differentiation and lactation and the composition appeared to remain constant. In the case of one ATPase MSS1, the level appeared to decrease early in involution, but this was probably due to less protein in this particular lane on the gel. Unfortunately, this antibody was a gift and we did not receive enough to repeat this result which therefore cannot be confirmed.

The increase in expression of the 26S proteasome components observed in the mammary gland was determined not to be dependent on lactogenic hormones. Studies in MECs showed no change in the expression of TBP1 regardless of whether the cells were undifferentiated, or induced to differentiate over a 12 day period. The expression profile of TBP1 in the mammary gland increased at the stage when the gland first starts to differentiate around day 10 gestation. The MEC used in this study, and all other available MEC lines, are derived from mid pregnant mice. In the case of KIM-2 cells, they were derived from day 12.5-13 gestation mice. It is possible that we observed no increase in TBP1 expression in this cell line as it was derived *in vivo* just beyond the stage when we first observed the increase in expression. These results determined that TBP1 was expressed in mammary epithelial cells. The expression profile of TBP1 in other mammary cell compartments remains unknown at present. Currently there are no antibodies available against TBP1 that are specific enough to give a definitive result. The availability of such an antibody may shed more light on the function of an increase in the 26S proteasome in the developing mammary gland. An antibody against TBP1 would allow the examination of expression of this protein in the developing mammary gland, within the specific cellular compartments and would also allow us to examine its role during proliferation and apoptosis. This would produce a more definitive role for this ATPase during gland development. A problem with the

majority of antibodies raised against these ATPases, all polyclonal antibodies, is that these proteins are so highly conserved that many of the antibodies are not very specific. In retrospect, it would have been useful to produce an antibody against TBP1 or perform *in situ* PCR to determine the expression of TBP1 in the myoepithelial and stromal compartments of the mammary gland. The expression peak in TBP1 and the 26S proteasome commences when the mammary gland starts to differentiate. This is also the stage when the gland first produces the milk protein β-casein. It is documented that by late gestation the mRNA for β-casein accounts for around 15-20% of the mRNA in the cell (Ball *et al.*, 1988). Following lactation the process of involution commences probably as a consequence of the accumulation of milk protein in the lumen. This protein acts in an autoregulatory loop to inhibit further milk protein synthesis (Feng *et al.*, 1995). Exactly how this process is bypassed during gestation is unclear. It is possible that because milk protein is not secreted until the very late stages of gestation then this feedback loop is not functional early in pregnancy. If this were the case then it would result in an accumulation of β-casein intracellularly. How this protein is removed from the cell is unclear. There are very few studies where degradation of milk protein has been examined. O'Hare and co-workers (1986) suggested that casein degradation was dependent on the lysosomal degradation system and that this degradation was highest when casein synthesis and secretion was least, and vice versa. These studies were performed using mammary explant cultures and therefore may not be directly relevant to the *in vivo* situation. In addition they do not entirely agree with our data as our increase was observed at the stage when the mammary gland begins to produce milk protein. More recently the proteasome has been described as containing a proteolytic activity capable of degrading β-casein. Inhibition of the three major activities of the 20S proteasome with a serine protease inhibitor had been observed to stimulate a caseinolytic-like activity in bovine pituitary or lens proteasomes (Pereira *et al.*, 1992). This proteolytic activity was seen to cleave β-casein into a 21kDa intermediate, which was then further processed to smaller peptides. This study demonstrated that under certain conditions the 26S proteasome has the ability to degrade milk protein. The caveat remains that this study was performed *in vitro* using purified 20S proteasomes, and many proteins can be

degraded in this situation. Whether these *in vitro* substrates are actually degraded in the same manner *in vivo* and with the 26S proteasome remains unknown. It may be possible that the increase in proteasome function observed in the mammary gland is due to degradation of milk protein, but other more probable theories exist. At the particular stage in development when TBP1 expression increases the mammary gland gains a specialised function and as a result a complex process of changes in gene expression and regulation are required. The increase in proteasome function may be a prerequisite for a cell to survive when it gains a more specialised function. It may be required to remove proteins that are cell type specific and thus allow the gland to switch from a proliferative state to differentiation. This functional progression may also result in an increased metabolism within the cell, which would require an increase in the systems that act to regulation the levels of protein production and degradation in the cell. Indeed the observation of downregulation of the 20S proteasome early in involution fits with this theory. It would seem highly probable that the proteasome may become a lesser requirement as the mammary gland is commencing its remodelling program. The major proteases involved in gland remodelling are the matrix metalloproteinases and the caspase, Interleukin-1β converting enzyme (ICE). It may also be assumed that following later stages in involution may show an increase in expression of the proteasome as the gland begins to proliferate to repopulate the fat pad. The further examination of proteasome function in range of developing tissues is required to determine if this is a general phenomenon or one that is specific to mammary gland development. Until recently, the major focus of proteasome research has been on the biochemical characterisation of the complex, and the study of yeast mutants of this pathway. When the bias of research transfers to examining proteasome function in mammalian systems, then the role of the 26S proteasome in developing tissues may become clearer.

Within this chapter two-hybrid analysis was performed on three ATPases to determine their organisation in the 19S regulatory complex. The results generated from this work initially appeared inconclusive. The assay was performed on three ATPases, TBP1, TBP7, and Sug2and no definitive interactions were observed between any of these proteins, which was unexpected. TBP1 in conjunction with the

Gal4 DNA binding domain was able to activate transcription alone, or with unrelated proteins in this assay. Therefore most of the results observed by this analysis related to the interaction of TBP1 pAS with each of the proteins tested. One weak interaction that could be intermittently detected was between Sug2 and TBP1, although this could only be confirmed in one direction due to TBP1 in the DNA binding domain vector transactivating alone. The failure to detect any interactions in this study was unexpected as it had been previously determined that the yeast homologue of S4 and murine MSS1 interacted using this assay (Gordon *et al.*, 1993) and therefore it was assumed that interactions could be detected between ATPases using this system. The underlying hypothesis at the start of this analysis was that all 6 ATPases may sit in a ring structure in the cap complex. Therefore it seemed probable that some of these ATPases should interact with one another. The results produced from the interaction assay may simply be a technical problem with the assay. The major technical problem associated with this technique is that the hybrid proteins are targeted to the nucleus, and the interactions may be dependent on post-translational modifications that take place in the endoplasmic reticulum, such as glycosylation and disulphide bond formation (Allen *et al.*, 1995). These modifications may not occur in this system, but this should only limit the use of two hybrid for screening against extracellular proteins and plasma membrane receptors. Proteins that cannot fold correctly in the cytoplasm or that require other modifications e.g. phosphorylation or acetylation by non-yeast proteins may also not be suitable for use in a two-hybrid screen. Sometimes interactions that occur commonly *in vivo* fail to be detected when tested using the two-hybrid system. The reason for this remains ambiguous, but may be due, in some cases to high level expression of the fusion protein being toxic to the reporter strain. Another reason for failing to detecting a signal is that the fusion proteins may not be stably expressed in yeast. The failure to detect an interaction in this study was a consequence of using the two-hybrid system for analysis, as recent biochemical studies have detailed interactions between ATPases that we could not conclusively detect. Technically, all the fusion proteins were expressed, although not at very high levels, therefore toxicity of the fusion can probably be eliminated. As interactions have been detected using yeast homologues of ATPases with this type of analysis, then it is possible that

the failure to discover any interactions using the murine ATPases was due to some modification requirement from a protein that is not expressed in yeast. Clearly a biochemical analysis is required to address this question.

Previous work with human TBP1 has shown that TBP1 can interact with both itself and with TBP7 (Ohana *et al.*, 1993). The two-hybrid system used above was slightly different from the one used in this study e.g. TBP1 was fused to a Gal4 DNA binding domain (amino acids 1-147, identical to that in pAS) and tested against TBP7 fused to a herpes virus VP16 activation domain. Mutation studies revealed that the first 100 amino acids of TBP1 were essential for interaction. Ohana and colleagues (1993) did not detect any transactivation activity from human TBP1 fused to the Gal4 DNA binding domain in the yeast two hybrid study. In contrast to this, they detected a transctivation activity for human TBP1 fused to Gal4 DNA binding domain co-transfected with a Gal_{UAS}-driven thymidine kinase or HIV promoter. This transactivation potential was seen to be promoter specific e.g. TBP1-Gal4 DNA binding domain did not drive strong expression of a mouse mammary tumour virus (MMTV) promoter. This transactivation potential was abolished by mutation of the P-loop and the helicase-like domain. The observation that human TBP1 does not activate transcription in yeast in the above study conflicts with the data generated in this study. The Gal4-DNA binding domains used were identical in both systems therefore the inability to detect binding in this study may be due to a technical problem with the particular yeast vectors and reporter strain used for the assay, or it is equally possible that TBP1 does display a transactivation potential. The results generated from biochemical studies are more concrete. These studies involve synthesising radiolabelled proteins from a reticulocyte lysate and performing a nitrocellulose binding assay, and have shown that S4 binds to MSS1, TBP7 binds to Sug1, and Sug2 binds to TBP1 (Richmond *et al.*, 1997). This study confirmed the previous work suggesting S4 and MSS1 interacted (Gordon *et al.*, 1993). These results from the biochemical assay determined that TBP1 and Sug2 associate tightly with one another but did not interact with any of the other ATPases analysed in this study. This is thought to suggest that these two subunits may be localised elsewhere in the regulatory complex. The interactions observed in the Richmond study suggest

that we may only have expected to observe an interaction between Sug2 and TBP1. The biochemical binding assay failed to detect an interaction between TBP1 and TBP7, similar to the results described in this thesis. These data conflict with those published by Ohana and colleagues (1993) who suggested that human TBP1 and TBP7 interacted using a yeast two hybrid assay. Richmond and co-workers (1997) addressed the question of whether all 6 ATPases can sit in a hexameric ring in direct contact with the proteasome α subunits. They suggested that a hexameric ring of approximately 45kDa proteins would extend beyond the edges of the central cylinder of the proteasome, and that this is difficult to visualise given the published crystal structure of the proteasome (Peters *et al.*, 1993, Groll *et al.*, 1997). Thus the concept of a ring of 4 central ATPases, with a further two located more peripherally may be the preferred model (Figure 4.7b). At present no further details on ATPase arrangement in the regulatory complex is available therefore there is still some confusion on whether all of these ATPases sit in a hexameric ring structure, the preferred theory previous to the publication of this work, or whether there is a central compartment of 4 ATPases.

The premise at the start of the two hybrid analysis was that all six ATPases sat in a ring structure within the 19S regulatory complex. The rationale behind the two hybrid analysis described in this chapter was based on a previous publication detailing an interaction between TBP1 and TBP7 (Ohana *et al.*, 1993). If this assumption had proved to be correct then we expected to be able to orientate all six ATPases in the complex from these studies. The inability to detect any interactions in this thesis has been validated with a recent publication from the laboratories of Richmond and colleagues (1997), and the elucidation of the crystal structure of the 20S proteasome (Groll *et al.*, 1997). From this later work it is now apparent that no interactions occur in any of the proteins examined herein with the exception of TBP1 and Sug2. More comprehensive studies are required to resolve the biochemical structure of this complex.

Recent data has also served to heighten the debate as to whether these ATPases merely function as components of the proteasome or whether they can play

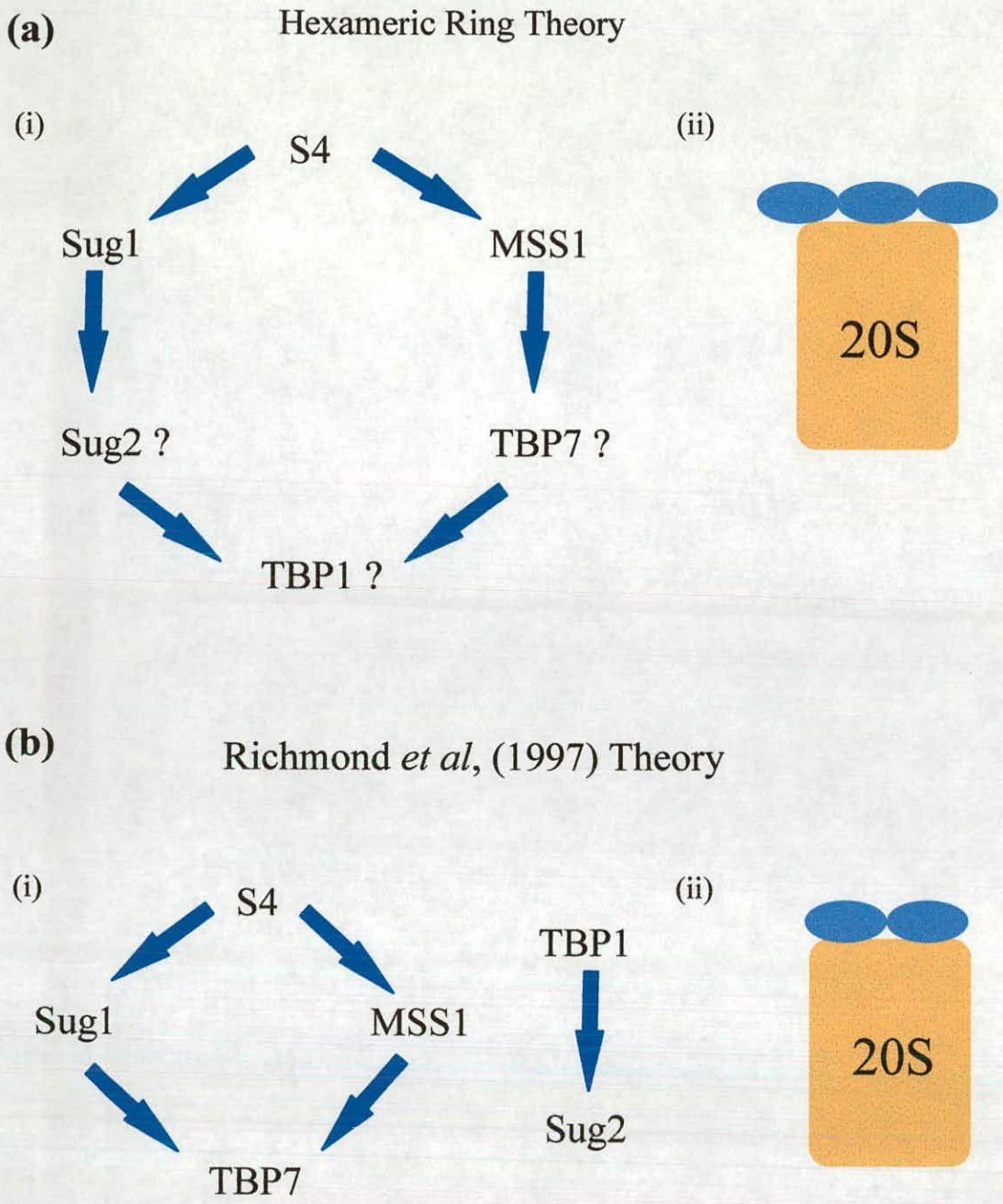


Figure 4.7b Theories on the structure of the 19S regulatory complex.
(a) Original theory was based on all 6 ATPases forming a ring structure. (ii) Face on view of proposed structure of 26S proteasome with 6 ATPases sitting in a ring.
(b) The second theory is based on the observations from the crystal structure of the yeast 20S proteasome. It indictates that if all 6 ATPases sat in a ring this would be too large to sit on top of the 20S core. (ii) Richmond and colleagues (1997) proposed that 4 ATPases sit in a central ring with two outlying ATPases.

a transcriptional role. In general it is assumed that some of the transcriptional phenotypes observed in yeast strains mutant in ATPase subunits may by due to a proteasomal defect. For example, the proteasome may be involved in proteolytically processing or degrading a transcription factor. Inhibition of this pathway may result in either an inhibitory effect on transcription where this complex is involved in activating a transcription factor, or in the stimulation of transcription where the proteasome is involved in degrading a transcription factor. Data from studies with TBP1 and Sug1 suggest that if any of these proteins play a transcriptional role, then these are the two most likely candidates. Ohana and colleagues (1993) showed a stimulatory activity of TBP1 on a variety of different promoters. Sug1 has been shown to contain an ATPases activity that was stimulated by synthetic RNAs and mRNA-type RNAs (Makino *et al.*, 1997). This would suggest that Sug1 may play a role in RNA metabolism as well as proteolysis. Again this effect may be due to the possibility that some mRNA-binding proteins undergo conformational changes induced by interactions with mRNAs, which may be targets of Sug1 proteasome mediated proteolysis. Fraser and colleagues (1997) have suggested that Sug1 exhibits a 3'→5' DNA helicase activity, and this was confirmed with the identification of Sug1 interacting with the XPB subunit of the repair/transcription factor TFIIH (Weeda *et al.*, 1997). Only a small proportion of cellular Sug1 was identified bound to the nucleotide excision repair helicase XPD in complex with TFIIH. These data have pointed to a more universal role for Sug1, and suggested a link between the basal transcription initiation/repair factor and the cellular machinery implicated in protein remodelling and degradation. Given that the six ATPases involved in protein degradation are highly structurally conserved, then I suggest that it is highly probably that other ATPases may play a transcription role in the cell. Paramount to the study of a transcriptional role is the differentiation between the proteolytic and transcription effect. At present, there is scepticism around any transcriptional function as this response may represent a function of disruption of protein degradation. Assigning functions to the different regions contained within these proteins should lead to a better understanding of the structure and function of the 26S proteasome, and also of any additional roles these ATPases may play.

Chapter 5:

Analysis of the Effect of a Proteasome Inhibitor on KIM-2 Mammary Epithelial Cells

5.1 Introduction

The aim of the studies reported in this chapter was to determine the effect that inhibition of the ubiquitin dependent 26S proteasome pathway had on KIM-2 mammary epithelial cells. We have previously observed that levels of the 26S proteasome increase in the mammary gland during the later half of gestation. It is well documented that the mammary gland responds to a massive proliferative signal in the early stages of pregnancy and it was hypothesised that the increase in the 26S proteasome may be essential for the maintenance of a low level of apoptosis during pregnancy. To address this question we examined the effect that proteasome disruption had on a mammary epithelial cell line undergoing proliferation. A synthetic peptide aldehyde inhibitor was used to block proteasome function. The recent interest in ubiquitin-dependent protein degradation has led to the identification of several hydrophobic peptide aldehydes which act to block peptidase sites within the 20S proteasome and thus can be used to study this pathway (Rock *et al.*, 1994). These inhibitors include N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLnL), N-acetyl-L-leucinyl-L-leucinyl-methional (ALLM), N-carbobenzyoxyl-L-leucinyl-L-norvalinal (MG115), and N-carbobenzyoxyl-L-leucinyl-L-leucinyl-leucinal (MG132). These peptides bind in the active sites formed by two related neighbouring β subunits. The three amino acids of the inhibitor (P1-P3) are in extended conformation and form hydrogen bonds with sites on either side of the hydrophobic central channel. This blocks the active site flanking residues Val20, Thr21, and Met22 and on the other side of the hydrophobic cleft Ile45, Ala46, Gly47, Leu48, and Val49 (Ditzel *et al.*, 1997). Crystallographic studies revealed that the inhibitors bind in a β sheet conformation between an extended loop between the β strands S2 and S3 of the β subunits. ALLnL and ALLM also function in inhibiting calpains which are cysteine proteases, and therefore are more commonly referred to as calpain inhibitor I and calpain inhibitor II respectively. Their inhibitory activity on calpains is similar whereas calpain inhibitor II has a stronger inhibitory effect on the 20S proteasome than does calpain inhibitor I. In contrast to this MG115 and MG132 have more specific effects on proteasome inhibition, and these effects are at

least 3 fold more potent that those of the calpain inhibitors (Rock *et al.*, 1994).

The vast majority of the initial studies analysing these aldehyde inhibitors focused on the inhibitory effect on the 20S proteasome itself. Recently it has been shown that these inhibitors are less potent against the 26S proteasome. This was thought to be due to the active sites of the proteasome being less accessible when they are incorporated into the 26S proteasome although it has been shown that these peptides clearly inhibit the 26S proteasome function *in vitro*. They have been observed to block the presentation of ovalbumin on MHC Class I which has been shown to require ubiquitin conjugation (Michalek *et al.*, 1993). These peptide aldehyde inhibitors have been demonstrated to have no obvious toxicity to cells as treatment at high concentrations for several hours shows no obvious necrosis within the cell population treated. They are also documented to be able to enter mammalian cells and inhibit proteasome function *in vivo* in a reversible manner. Initial studies of these inhibitors in yeast showed that following removal of MG132, the degradation of pulse-labelled proteins returns to normal rates (Lee *et al.*, 1996). This reversible effect also argues that the reduction of proteolysis observed with these inhibitors is due to inhibition of the proteasome and not to a toxic action of the inhibitor. Lee and colleagues (1996) noted in yeast that the inhibitory effect of MG132 was lost after 5 hours incubation. This was reversed by the addition of more inhibitor suggesting that these peptides were metabolised much more rapidly in yeast, as they are known to block degradation for longer periods in mammalian systems.

More recently a newer class of compound has been described which acts to block proteasome function. The antibiotic lactacystin, which is cleaved to the more potent β-lactone has been attributed with blocking the three major activities of both the 20S and 26S proteasome (Craiu *et al.*, 1997). Unlike some of the peptide aldehyde inhibitors, β-lactone does not inhibit any other protease pathways and its effects are irreversible. β-lactone binds in close proximity to the N-termini of all 14 β subunits (Ditzel *et al.*, 1997) and blocks the degradation of proteins with similar

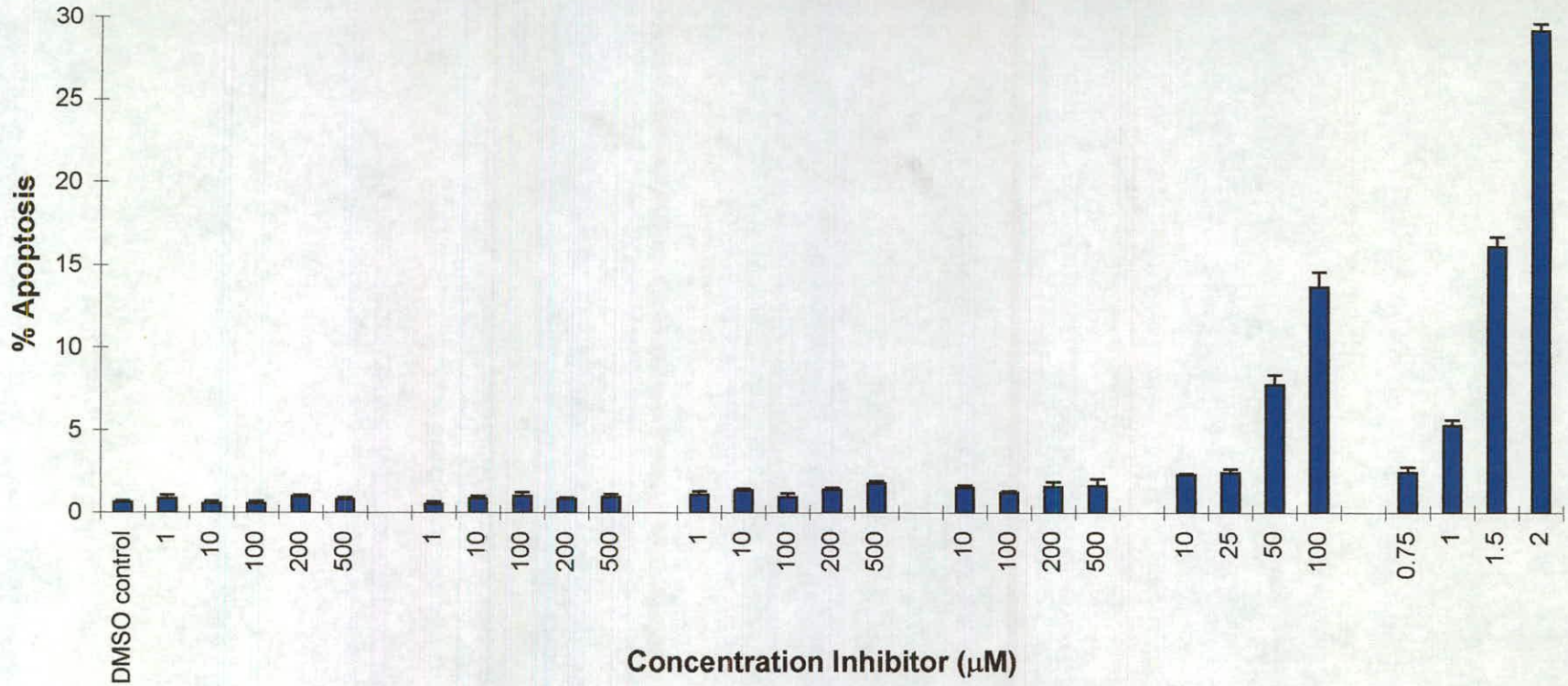
kinetics to MG132. Dick and co-workers (1997) have shown that only β-lactone can enter cultured mammalian cells, therefore the conversion of lactacystin to β-lactone is rate limiting for its inhibitory effects. The availability of these inhibitors has proven a useful tool in the analysis of the regulation of proteolysis within the cell and more generally has expanded our knowledge on a number of processes in which the rapid turnover of proteins plays an important regulatory role.

In this chapter I have used the peptide aldehyde inhibitor MG132 to specifically block proteasome function in mammary epithelial cells. The effects of proteasome inhibition were analysed by morphological assessment of cell death by staining cells with acridine orange and examination by fluorescence microscopy. Apoptotic cells are generally distinguished by characteristic morphological and biochemical changes such as cell shrinkage, chromatin condensation, cell fragmentation and chromosome DNA cleavage at internucleosomal sites (Ellis *et al.*, 1991). A flow cytometric Annexin V assay was performed to specifically quantitate the proportion of cells undergoing apoptosis following proteasome inhibition. Annexin V binds to phosphatidylserine (PS) which flips from the inner to the outer surface of the cell membrane early in the apoptotic cascade. By counterstaining cells with propidium iodide, which will only enter if the cell membrane is disrupted, apoptotic and necrotic cells can be distinguished. This assay can therefore be used to detect cells undergoing one of the early changes characteristic of apoptosis. Apoptotic changes within a cell are a transient event taking between 15 minutes to 1 hour (Wyllie *et al.*, 1980). After a few hours, apoptotic cells undergo a later non-specific deterioration as their membrane permeability increases. At this stage the apoptotic cells cannot be distinguished from cells undergoing primary necrosis. As a consequence of this, I analysed apoptotic changes at specific time points to quantitate the apoptotic events occurring at each particular time.

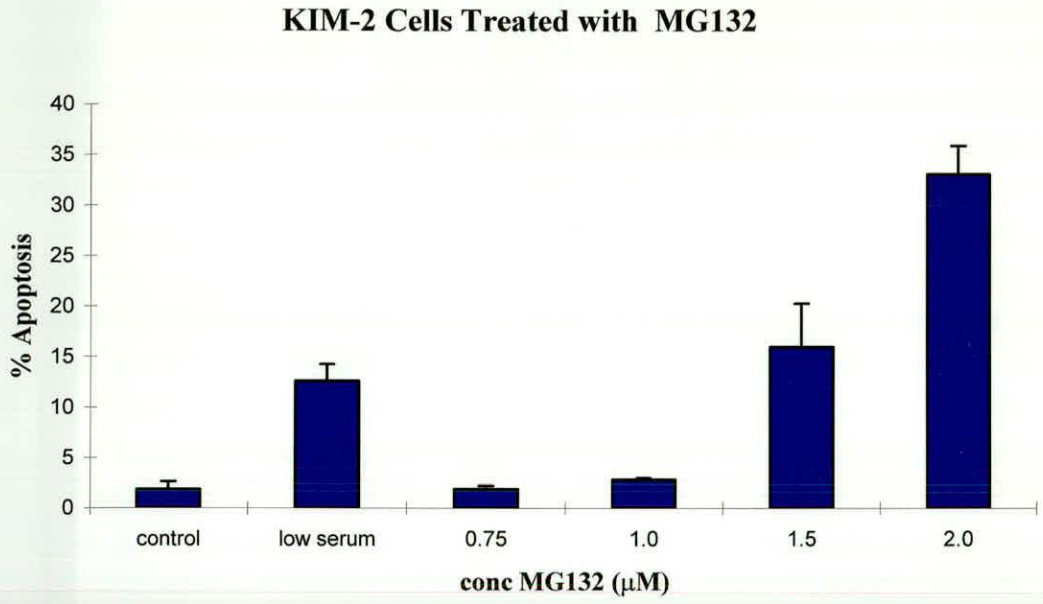
5.2 Induction of Apoptosis by Proteasome Inhibition

Proteasomes contain a number of distinct proteolytic activities: these include a chymotrypsin-related activity, a trypsin-related activity, and a peptidylglutamyl-peptide hydrolysing activity. The peptide aldehyde inhibitor of the proteasome, MG132 selectively inhibits the chymotrypsin-like activity of the complex resulting in inhibition of this degradation pathway (Rock *et al.*, 1994). To investigate the role of the proteasome in apoptosis, we examined the effect of MG132 as well as other protease inhibitors on KIM-2 mammary epithelial cells. Figure 5.1 provides evidence that inhibition of the proteasome in mammary epithelial cells (MEC) induced apoptosis. The results shown were determined from morphological analysis of acridine orange stained cells; apoptosis was identified by the presence of cytoplasmic shrinkage, and nuclear fragmentation. The cysteine/serine protease inhibitor Leupeptin, the aspartic protease inhibitor Pepstatin, and the serine protease inhibitor PMSF, did not induce apoptotic cell death at high concentrations over a 24 hour period (Grimm *et al.*, 1996, Sadoul *et al.*, 1996, and Drexler, 1997). The lysosomal cathepsin inhibitor, E64d ester did not induce apoptosis in MEC. In contrast to this, calpain II inhibitor (ALLM) induced a low level of apoptosis (12%) at a high concentration (100μM) over the course of the experiment. 2μM of MG132 induced >25% death over an identical time period. ALLM is well documented to cross-react with the proteasome in addition to calpains (Rock *et al.*, 1994). E64d, which has cross-reactive inhibitory activity on calpains did not induce apoptosis therefore it seems highly likely that the death observed with high concentrations of ALLM treatment is due to its inhibitory activity on the 26S proteasome rather than the inhibition of calpains. Figure 5.2 shows MG132 treated KIM-2 cells stained with acridine orange, showing the characteristic features of apoptosis. In the following experiments KIM-2 cells were routinely treated with 5μM MG132 unless otherwise stated. Titration of MG132 demonstrated that there was no major difference in the level of death induced by either 2μM or 5μM MG132, and no enhanced necrotic effects were observed at the higher concentration as shown by an Annexin V assay,

Figure 5.1. Effect of a range of protease inhibitors on KIM-2 mammary epithelial cells. The protease inhibitors PMSF, Leupeptin, Pepstatin A, E64d, Calpain II Inhibitor, and MG132 were added to media at various concentrations for 24 hours. Cells were harvested and fixed for staining with acridine orange. The percentage death is shown as the mean of 3 independent experiments ± sem.


% Apoptosis
30
25
20
15
10
5
0
DMSO control
1
10
100
200
500
1
10
100
200
500
1
10
100
200
500
10
100
200
500
10
25
50
100
0.75
1
1.5
2
Concentration Inhibitor (μM)
PMSF
LEUPEPTIN
PEPSTATIN A
E64d
CALPAIN II INHIBITOR
MG132

(a)



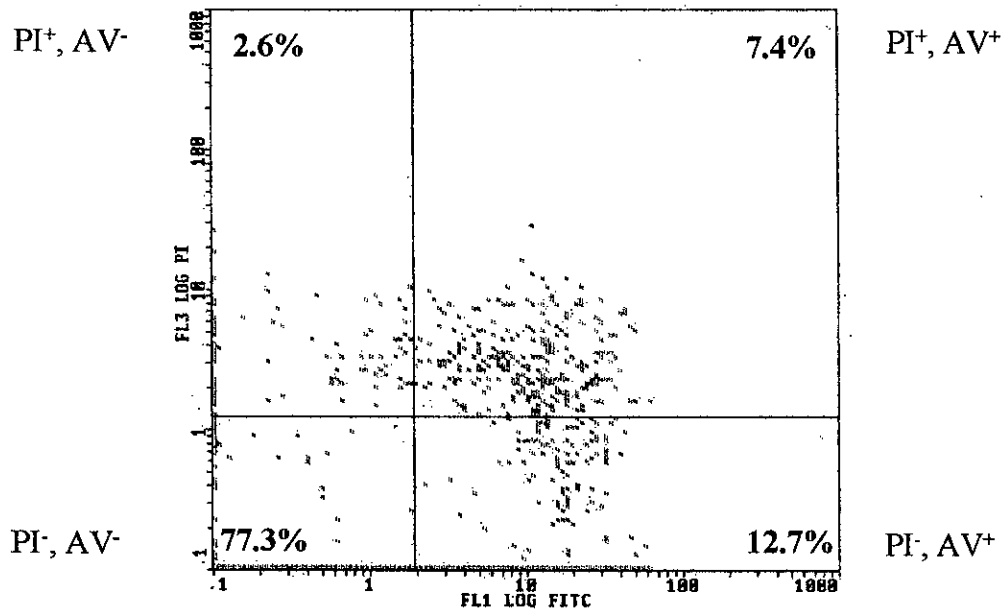
(b)

Figure 5.2 Effect of Proteasome Inhibition on KIM-2 Mammary Epithelial Cells. (a) KIM-2 cells grown to confluence were treated with a range of concentrations of MG132 for 24 hours. As a positive control cells were also serum and growth factor starved (low serum) to induce apoptosis. Cells were harvested and fixed prior to staining with acridine orange. Cell death was assessed morphologically by flourescence microscopy. The results represented are the mean of three individual experiments ± sem. 500 cells were counted for each sample presented. (b) Acridine orange stained KIM-2 cells were assessed for apoptosis by the presence of cytoplasmic shrinkage and nuclear condensation. Examples of apoptotic cells are identified by the letter A's.

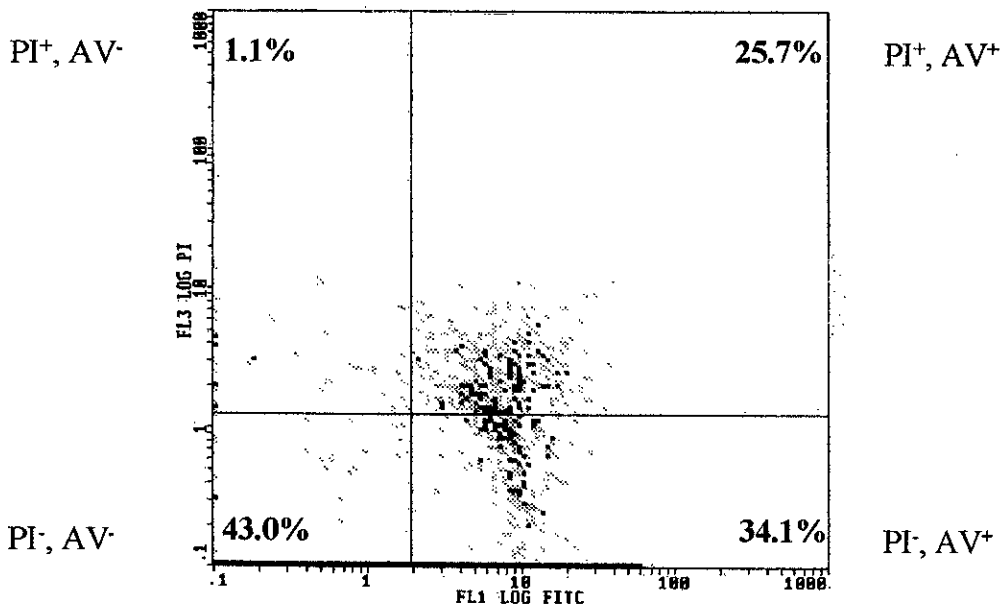
the principles of which are described below. As previous groups had published treatments of cells in culture with concentrations of between 20μM and 50μM MG132, we chose to use the higher concentration (5μM) of inhibitor to ensure we were inhibiting protein degradation at as effective a level as possible.

A flow cytometric Annexin V assay was performed to allow a faster and more accurate quantitation of apoptosis following inhibition of the 26S proteasome. This assay also allows the processes of apoptosis and necrosis to be distinguished. Examples of flow cytometry data are shown in Figure 5.3a. Annexin V and PI staining are shown for a control and MG132 treated sample. The lower left quadrant displays the viable unstained cells, with the upper left being PI positive (necrotic) cells. The lower right quadrant contains Annexin V (apoptotic) positive cells, with the upper right quadrant containing dual stained cells (necrotic and late apoptotic cells). When this assay is performed over a range of time, the population of apoptotic cells can be seen to later become positive for PI as well as Annexin V. KIM-2 cells are grown as a monolayer and thus are harvested from the flask by trypsinisation. This process results in the rupture of a number of cell membranes, increasing the background level of Annexin V and PI positive cells. This can be seen in the control sample where 7.4% of the cells are staining for both Annexin V and PI. By contrast the level of dual staining increases to 25.7% in the MG132 treated sample suggesting that this population now contains cells which are necrotic, in conjunction with cells which are showing signs of late apoptosis. Figure 5.3b shows data from KIM-2 cells treated with MG132, and harvested over a 24 hour time period. These results show that apoptotic cells can be observed within 4 hours of MG132 treatment. The death response observed appeared to be bi-phasic e.g. a peak in apoptotic cells was observed within the first 8 to 10 hours and then another between 18 and 20 hours. This may be a function of the MEC progression through particular stages of the cell cycle. This result also showed an increase in the proportion of double stained cells with time, suggesting that cells which had undergone apoptotic responses later began to stain positive for PI as well as Annexin V.

Figure 5.3a. Flow Cytometric Annexin V assay. Examples of control and MG132 treated samples. Cells were treated with either DMSO or 5μM MG132 for 24 hours. Samples were harvested and 10^5 cells stained with Annexin V (AV) and Propidium Iodide (PI) using an R&D Systems Apoptosis Detection Kit. The data is shown as the percentage viable cells (no stain, in the lower left quadrant), Annexin V positive (apoptotic, in the lower right quadrant), PI positive (necrotic, in the upper left quadrant), and Annexin V and PI positive (necrotic and late apoptotic, in the upper right quadrant). The data is subsequently expressed as the percentage of Annexin V positive cells e.g. 12.7% for controls and 34.1% for cells treated with 5μM MG132.



Control KIM-2 Cells



KIM-2 Cells + MG132 24 Hours

Control KIM-2 Cells

KIM-2 Cells + MG132 24 Hours

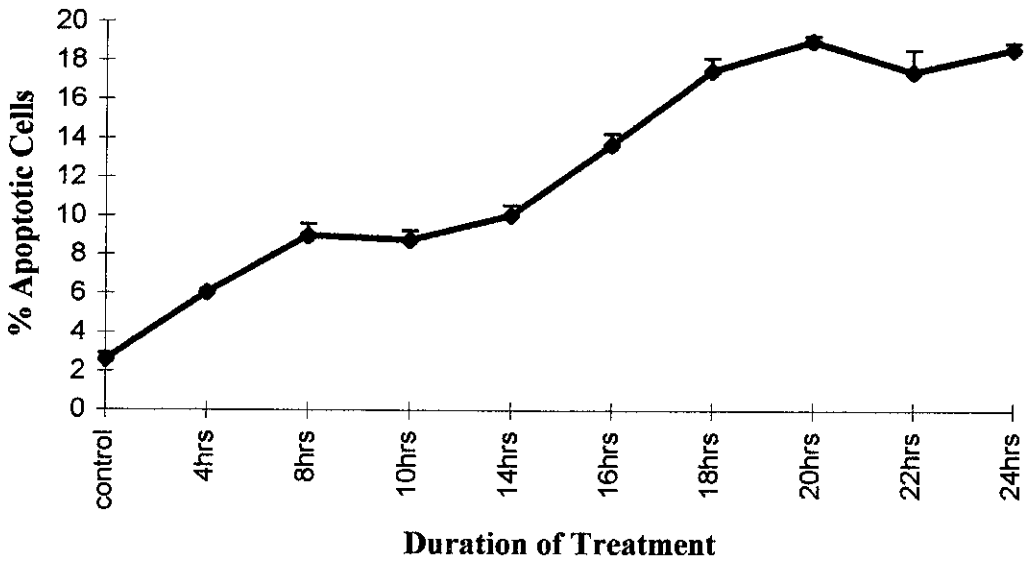
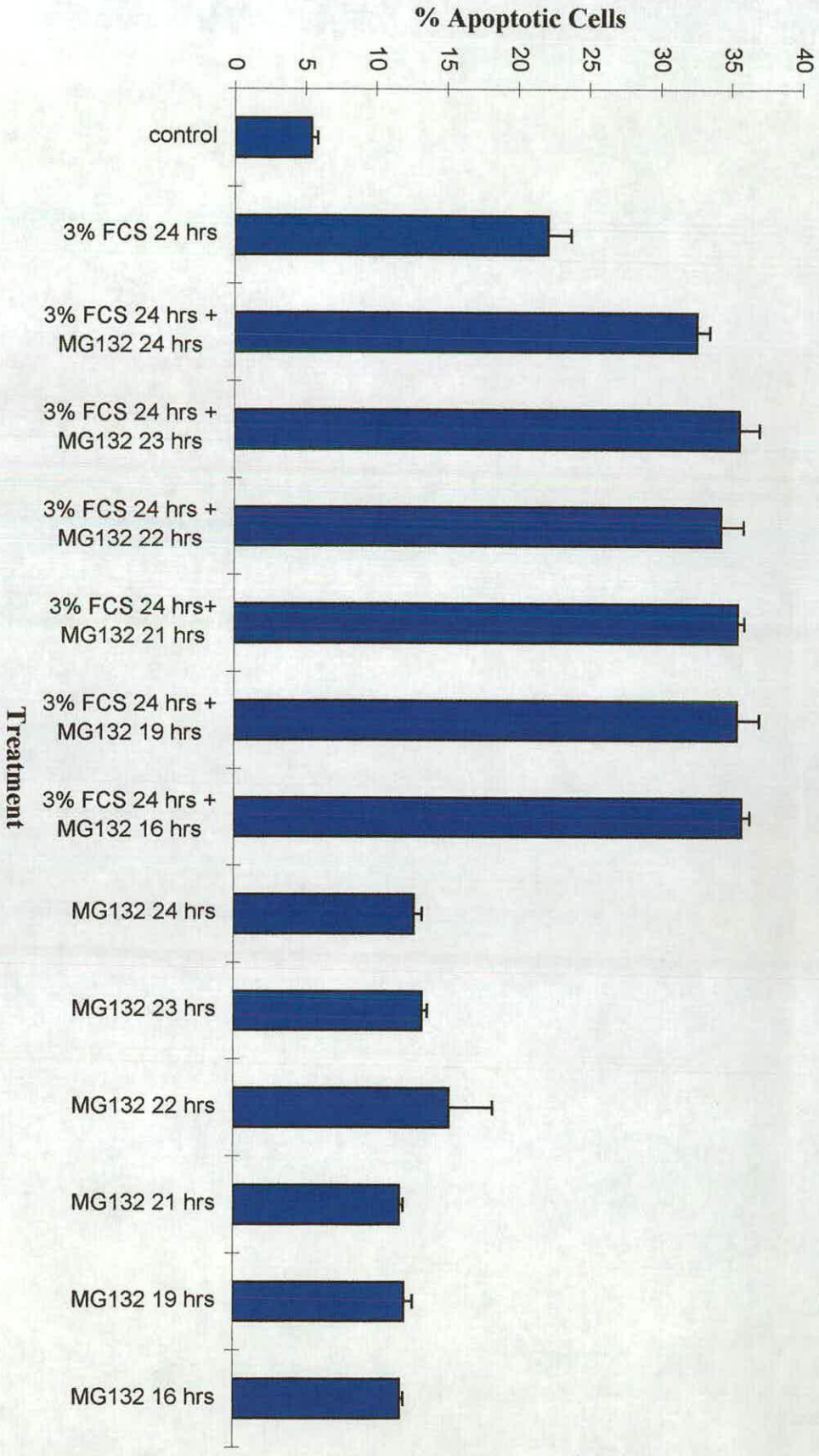


Figure 5.3b Annexin V time-course on KIM-2 Cells. KIM-2 cells were grown to confluence and treated with 5μM MG132 over a 24 hour period. Samples were harvested and an Annexin V assay was performed. The results shown are the mean of three experiments ± sem, and are expressed as the percentage of Annexin V positive cells.

Figure 5.4a. Can Proteasome Inhibition protect from Apoptosis? Confluent KIM-2 cells were treated with full maintenance media containing 5μM MG132 at 0, 1, 2, 3, 5, and 8 hours or in media containing only 3% FCS. All samples were harvested at 24 hours and used as controls for either treatment alone. Test samples were induced to die by treatment with 3% FCS for 24 hours. At time 0, 1, 2, 3, 5, and 8 hours into the serum starvation, 5μM MG132 was added to the cells. Samples were harvested at 24 hours and an Annexin V assay was performed. Results shown are expressed as the mean of three experiments ± sem.

% Apoptotic Cells
70
60
50
40
30
20
10
0
control
3% FCS 24 hrs
3% FCS 24 hrs+ MG132 24 hrs
3% FCS 24 hrs + MG132 23 hrs
3% FCS 24 hrs+ MG132 22 hrs
3% FCS 24 hrs + MG132 21 hrs
3% FCS 24 hrs + MG132 19 hrs
3% FCS 24 hrs+ MG132 16 hrs
MG132 24 hrs
MG132 23 hrs
MG132 22 hrs
MG132 21 hrs
MG132 19 hrs
MG132 16 hrs
Treatment


% Apoptotic Cells
0
5
10
15
20
25
30
35
40
Treatment
control
3% FCS 24 hrs
3% FCS 24 hrs + MG132 24 hrs
3% FCS 24 hrs + MG132 23 hrs
3% FCS 24 hrs + MG132 22 hrs
3% FCS 24 hrs+ MG132 21 hrs
3% FCS 24 hrs + MG132 19 hrs
3% FCS 24 hrs + MG132 16 hrs
MG132 24 hrs
MG132 23 hrs
MG132 22 hrs
MG132 21 hrs
MG132 19 hrs
MG132 16 hrs

At present it appears that the response to proteasome inhibition may depend on the system chosen for analysis. Recent results have suggested that proteasome inhibitors are capable of inducing cell death in some systems e.g. in lymphoma cells (Tanimoto *et al.*, 1997), Molt 4 cells (Shinohara *et al.*, 1996), neurons (Lopes *et al.*, 1997), and HL60 cells (Drexler, 1997), whilst other results suggest that inhibition of the proteasome can prevent apoptosis e.g. in thymocytes (Grimm *et al.*, 1996), and sympathetic neurons (Sadoul *et al.*, 1996). In KIM-2 cells we have shown that proteasome blockage induces an apoptotic response. In order to address whether proteasome inhibition could also play a protective role in mammary epithelial cells, KIM-2 cells were serum and growth factor (GF) starved to induce an apoptotic response. These cells were also treated with 5μM MG132 at the onset of serum and GF withdrawal and up to 8 hours following this death stimulus. Annexin V staining determined that MG132 could not protect from apoptosis induced by GF withdrawal (Figure 5.4a). This experiment was repeated using a lower concentration of MG132 (1.5μM) which induced less death and a similar result was obtained (Figure 5.4b). The effects on death observed with both treatments appeared to be additive. These experiments suggested that the proteasome is not involved in degrading and/or processing a protein that is essential for driving apoptosis induced by GF and serum starvation.

6.3 Induction of Apoptosis by Proteasome Inhibition Displays Cell Cycle Dependence

The differences observed in response to proteasome inhibition were thought to be due to differences in the cell cycle status of the cell lines examined. To address this question KIM-2 cells at different stages of confluence were treated with MG132 and death assessed morphologically by acridine orange staining. Cells were treated in this manner to represent samples that contained progressively less cells actively traversing the cell cycle. Cells were treated 4, 24, 48, 72, and 120 hours following

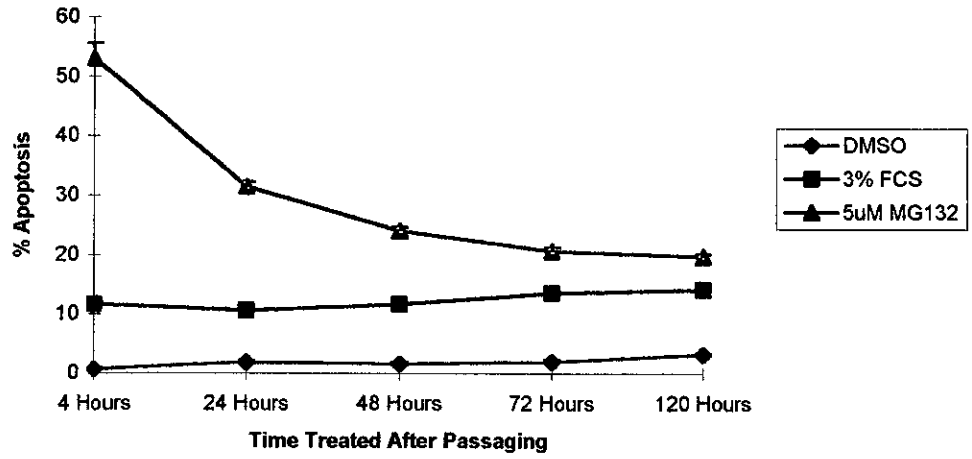
passaging with MG132 over a 24 hour period. By 72 hours KIM-2 cells were reaching confluence. These results showed that in cells treated 4 hours following passaging, MG132 treatment over 24 hours induced 50% death (Figure 5.5a). The level of death observed decreased as the cells became more confluent, and this remained at a minimum of around 20% in the samples treated from 72 hours onwards. These results showed a baseline level of 20% apoptosis in cells that were almost entirely confluent. This would suggest that the apoptosis induced by proteasome inhibition had a dependency on the cells being within the cell cycle. This was shown not to be the case for serum and growth factor starvation of the cells. 10-15% death was observed with this death stimulus regardless of the confluence status of the cells. To further this observation Annexin V staining was performed on KIM-2 cells that had been differentiated for 12 days in the presence of prolactin and dexamethasone and thus should be effectively out of cycle. Death was seen to occur in these cells although at a much lower level than that observed in undifferentiated cells (Figure 5.5b). These results together suggest that cell proliferation is a major component of the sensitivity to proteasome inhibition although not the sole determinant, as a low level of death was observed in both differentiated and confluent cells. These results could also explain the previous conflicting results; apoptotic death induced by proteasome inhibition can be observed in actively proliferating cells as opposed to terminally differentiated cells.

To determine if the apoptotic response observed as a consequence of proteasome inhibition was entirely dependent on a cell traversing the cell cycle, we performed experiments that involved blocking MEC in either G1/S phase with Hydroxyurea, which inhibits the production of bases for DNA synthesis, or G2/M with the spindle poison Nocodazole. The blocking effect of these drugs were optimised for KIM-2 cells revealing that 1mM Hydroxyurea treatment for 24 hours resulted in approximately 65-70% cells in G1/S phase. Nocodazole treatment (50ng/ml) blocked approximately 75% of cells in G2/Mitosis. Cells were blocked for one cell cycle (approximately 24 hours) and then medium was replaced with fresh medium containing either more Hydoxyurea or Nocodazole supplemented with 5μM MG132. The rationale of this experiment was to determine if apoptosis could be

induced in a cell population that was not actively progressing through the cell cycle. Unfortunately, the results obtained were not conclusive. Hydroxyurea is relatively toxic to the cells if left in contact for 48 hours even at concentrations as low as 1mM. This resulted in an enhanced level of background death precluding analysis of any real response, which could not be differentiated from the toxic effect of the drug on the cells. In contrast to this, Nocodazole was not toxic to the cells when left in contact for 48 hours, but the kinetics of blockage changed from 24 hours to 48 hours. At 24 hours we generally obtained 75% G2/M phase block, but at 48 hours this had reduced to 49% suggesting that there was some leakage from this G2/M block. The Annexin V assay showed approximately 40-50% cells were in the early stages of apoptosis (Figure 5.5c). As only approximately 50% of the cells had remained blocked in G2/M this may suggest that the death observed was from the cells that were no longer effectively blocked. These drugs did not block 100% of the cells and therefore it was difficult to interpret these results.

The results described above suggested that the effect of proteasome inhibition is dependent on cell cycle status. This was not unexpected as the proteasome is well documented to be involved in degrading proteins involved in cell cycle control. Experiments were therefore performed to determine if KIM-2 cells were undergoing apoptosis from a specific point in the cell cycle as a consequence of MG132 treatment. To achieve this cells were synchronised at particular stages of the cell cycle prior to treatment with MG132 and apoptosis analysed over a 24 hour time period. Figure 5.6a shows the morphological appearance of synchronised cells. Cells treated with Hydroxyurea and therefore blocked in late G1 appeared more elongated than unsynchronised control cells. In contrast to this, Nocodazole treatment caused a more dramatic change in the cells. KIM-2 cells rounded up and some detached from the plastic when blocked in G2/mitosis. Upon removal of these drugs and culture for 24 hours in drug free medium, the cells morphologically appeared very similar to unsynchronised control cells suggesting that these treatments were reversible and had no long-term toxic effects to the cells. Cells

(a)



(b)

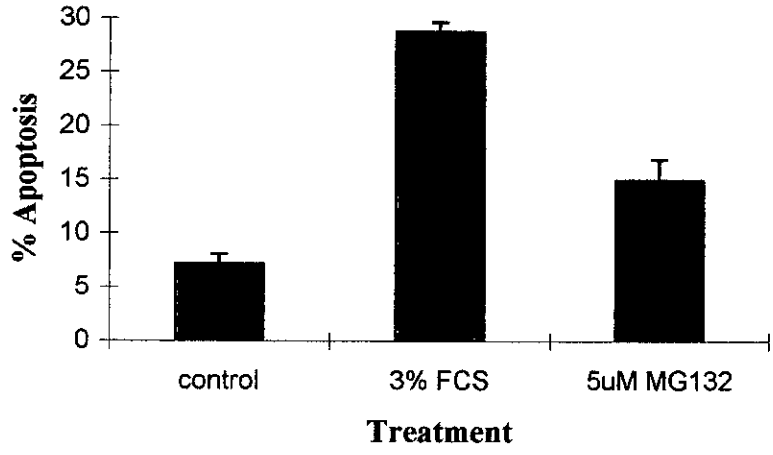


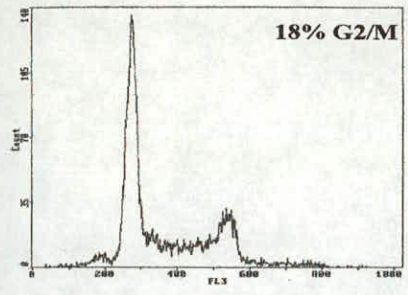
Figure 5.5 (a) Apoptosis Analysis in a Proliferating Cell Population. KIM-2 cells were passaged and treated with either vehicle alone (DMSO), serum and growth factor starved (3% FCS), or 5μM MG132 at 4, 24, 48, 72, and 120 hours after passaging. Cells were treated for 24 hours and an Annexin V assay performed. Results are shown as the percentage of Annexin V positive cells, and are expressed as the mean of three experiments ± sem.

(b) Effect of Proteasome Inhibition on Differentiated KIM-2 Cells. KIM-2 cells were differentiated for 12 days in the presence of dexamethasone and prolactin prior to a 24 hour treatment with either 5μM MG132 or serum and growth factor withdrawal (3% FCS). Cells were harvested and an Annexin V assay performed. Results are expressed as the mean of three experiments ± sem and expressed as the percentage of Annexin V positive cells.

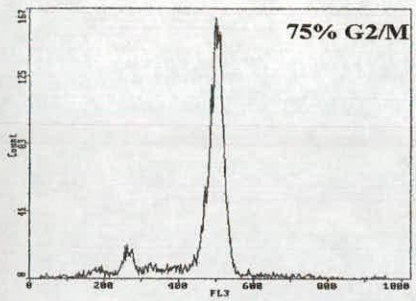
Figure 5.5c Effect of Proteasome Inhibitor on KIM-2 Cells which are not actively Progressing the Cell Cycle. (i) KIM-2 cells were cultured for a full 24 hours following passaging prior to treatment with 50ng/ml Nocodazole. Cells were harvested and fixed prior to staining with Propidium Iodide and analysis on a Coulter Epics flow cytometer. The examples shown represent unsynchronised KIM-2 cells, and either 24 or 48 hours synchronisation with Nocodazole.

(ii) KIM-2 cells were synchronised in G2/M phase for 24 hours using 50ng/ml Nocodazole. At 24 hours cells were either treated with addition of Nocodazole alone, or Nocodazole supplemented with 5μM MG132 for a further 24 hours. As controls, cells were treated with either Nocodazole or MG132 alone for 48 hours. KIM-2 cells were harvested and an Annexin V assay performed. Results are shown as the percentage of Annexin V positive cells, and as the mean of three experiments ± sem.

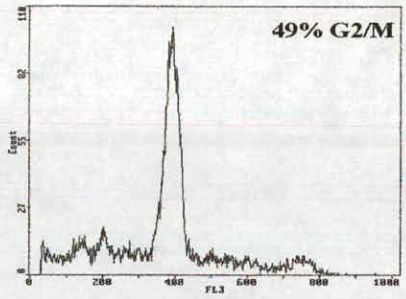
(i)



Unsynchronised KIM-2 Cells

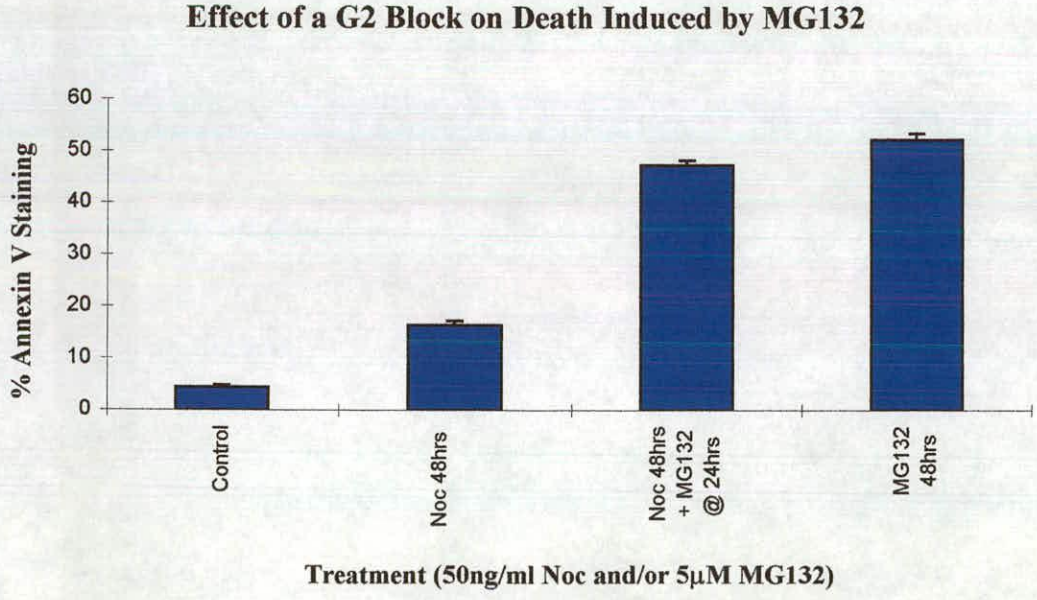


50ng/ml Nocodazole 24 hrs



50ng/ml Nocodazole 48 hrs

(ii)

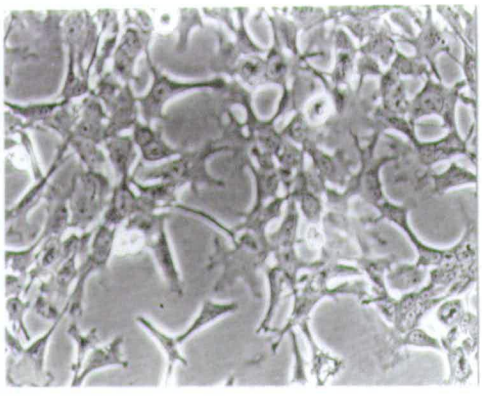


were blocked for 24 hours (approximately one cell cycle) and then the drug was removed and replaced with either maintenance medium (MM) or MM supplemented with MG132. Cells were harvested and stained with Annexin V for apoptosis analysis or fixed and stained with Propidium Iodide (PI) for cell cycle analysis. Results for cells blocked in G1/S phase showed that apoptosis was detectable within 2 hours of MG132 treatment, and this increased dramatically by 4 hours. In contrast to this, cells that had been blocked in G2/Mitosis did not begin to show a dramatic increase in Annexin V staining until 12-16 hours after treatment with MG132 (Figure 5.6b). Analysis of the cell cycle kinetics demonstrated that in cells released from a G1 synchronisation, MG132 treatment caused an accumulation of cells in the G1/S phase of the cell cycle. The cells appeared to be unable to progress any further than S phase (Table 5.1) compared to control cells (Table 5.3). The cell cycle profiles of these cells are shown in Figure 5.6c. KIM-2 cells synchronised in G2 prior to proteasome inhibition appeared to progress through G2/M but at a much slower rate than cells which were released from block into maintenance media (Tables 5.2 and 5.4, and Figure 5.6c). The MG132 treated cells did not begin to accumulate in G1/S phase until 10-12 hours following the onset of treatment. This corresponds to the onset of apoptosis in these cells. Similar to G1 synchronised cells, G2 synchronised cells were unable to progress any further than G1/S phase of the cell cycle. Taking the cell cycle and apoptosis results together it would appear that the 26S proteasome is implicated in functioning during mitosis as MG132 treated cells were less efficient at traversing this stage of the cell cycle than control cells. These results also detailed an absolute requirement for proteasome function at the G1/S phase of the cell cycle as the cells appeared to arrest and undergo apoptosis either during or following this stage in the cell cycle.

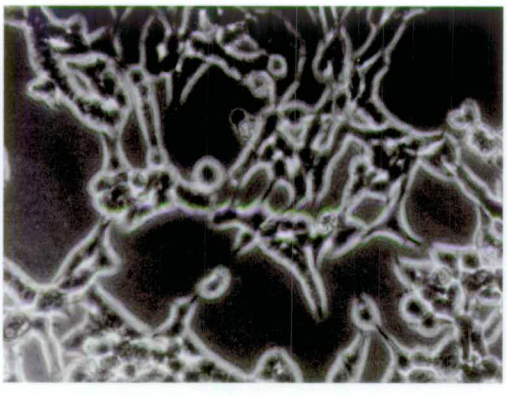
To ensure that KIM-2 cells were able to enter S phase prior to undergoing apoptosis we examined the levels of the cdk inhibitor $p27^{Kip1}$. p27 is elevated late in G1 and is one of a number of restriction points controlling the G1/S phase transition. Its association with the cyclinE/cdk2 complex catalyses its degradation by the 26S proteasome (Vlach *et al.*, 1997). It was assumed that if the cells were reaching this

Figure 5.6a. Cell cycle synchronisation of KIM-2 Cells. Phase contrast light microscopy of (i) unsynchronised proliferating KIM-2 cells, (ii) Proliferating KIM-2 cells synchronised in G1/S phase with 1mM Hydroxyurea for 24 hours, (iii) KIM-2 cells released from a Hydroxyurea block in full maintenance media for 24 hours, (iv) Proliferating KIM-2 cells synchronised in G2/Mitosis with 50ng/ml Nocodazole for 24 hours, (v) KIM-2 cells released from a G2/Mitosis block into full maintenance media for 24 hours. The scale bar represents 50 μm.

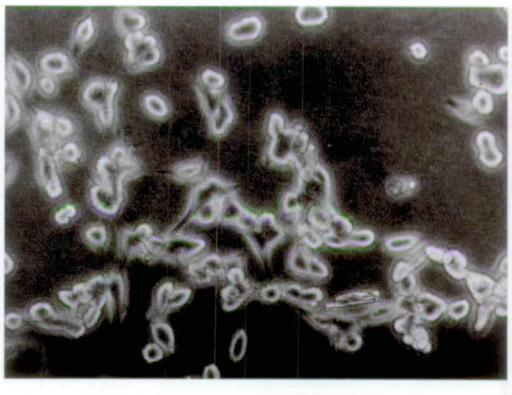
(i)



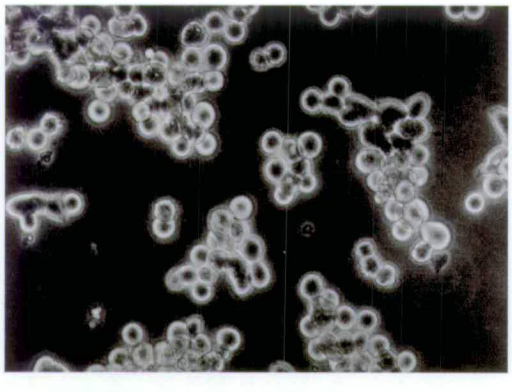
(ii)



(iii) (50μm ———)



(iv)



(v)

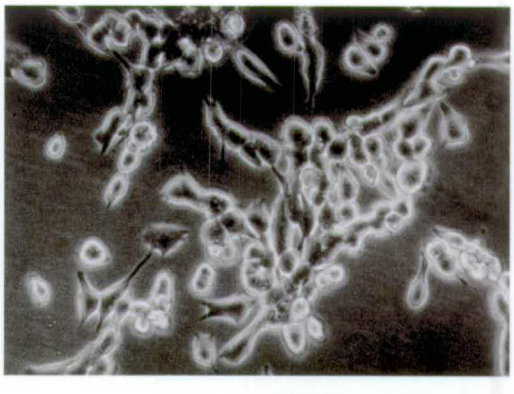


Figure 5.6b. Apoptosis induced from a G1/S phase arrest in KIM-2 cells treated with proteasome inhibitor. KIM-2 cells were passaged normally, and 24 hours later blocked with Hydroxyurea (1mM) or Nocodazole (50ng/ml) for 24 hours (approximate cell division time). Cells were washed to remove the blocking drugs and then treated with 5μM MG132 for a range of times. Cells were harvested and an Annexin V assay performed. Control samples are represented by apoptosis levels in cells treated with either of the blocking drugs for 24 hours and subsequently released into full maintenance media for a further 24 hours. Results shown are expressed as the percentage of Annexin V positive cells. The data is the mean of three experiments ± sem.

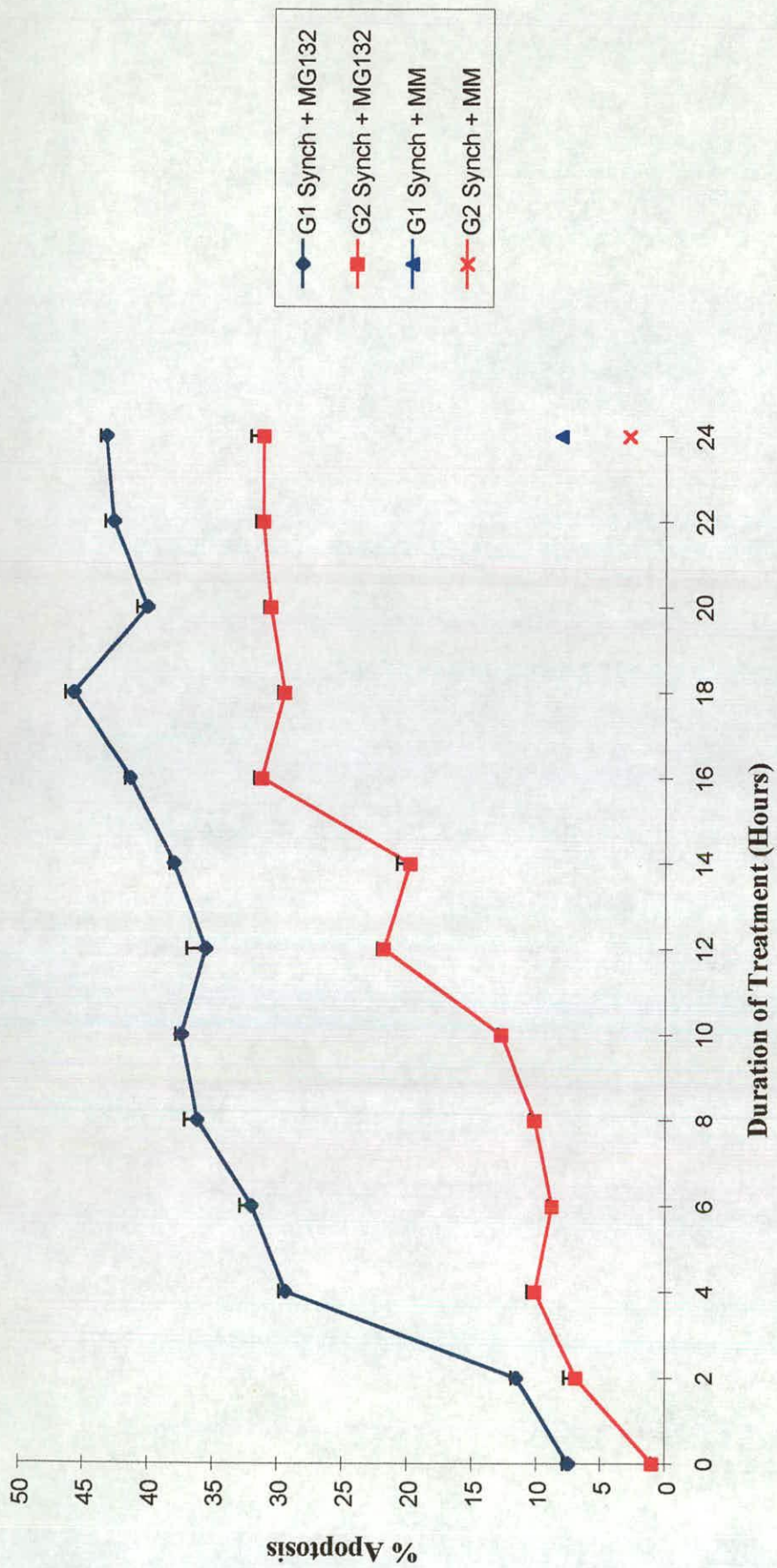
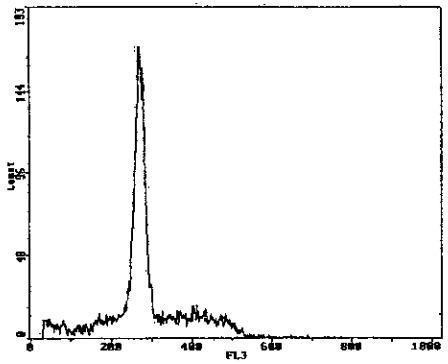
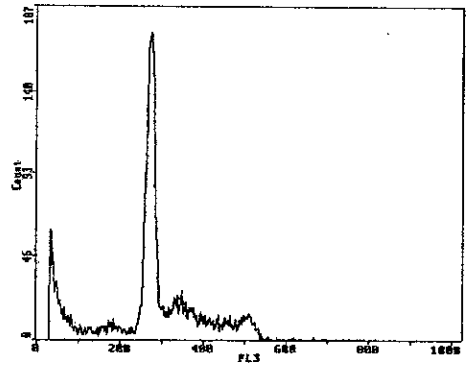

G1 Synch + MG132
G2 Synch + MG132
G1 Synch + MM
G2 Synch + MM
% Apoptosis
0
5
10
15
20
25
30
35
40
45
50
0
2
4
6
8
10
12
14
16
18
20
22
24
Duration of Treatment (Hours)

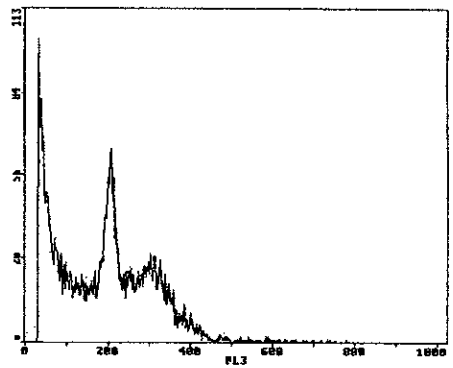
Figure 5.6c Effect of Proteasome Inhibition on Cell Cycle Distribution of KIM-2 Cells. KIM-2 cells were passaged normally and 24 hours later blocked in G1/S phase with 1mM hydroxyurea, or in G2/M phase with 50ng/ml Nocodazole. The blocking drug was then removed from the cells and they were subsequently placed in full maintenance media (MM) alone or supplemented with 5μM MG132 for a further 24 hours. KIM-2 cells were harvested and fixed prior to staining with propidium iodide for cell cycle analysis. Flow cytometry was then performed using a Coulter Epics cytometer. Three individual experiments were performed and the results shown are representative of these.



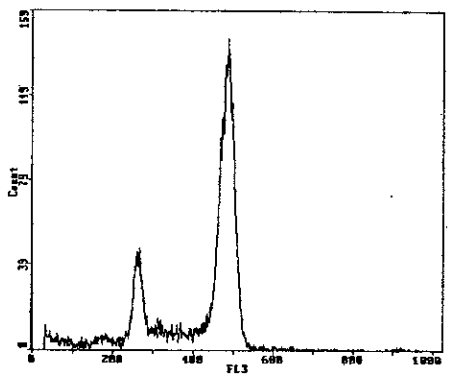
1mM Hydroxyurea 24 Hours



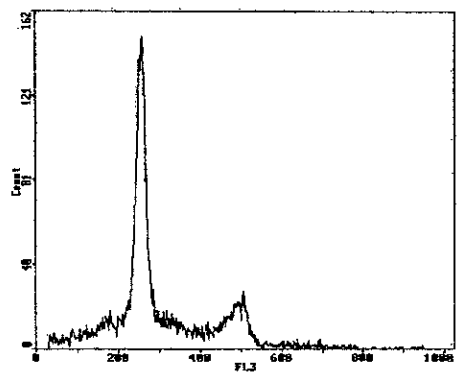
1mM Hydroxyurea 24 Hours + MM for a further 24 Hours



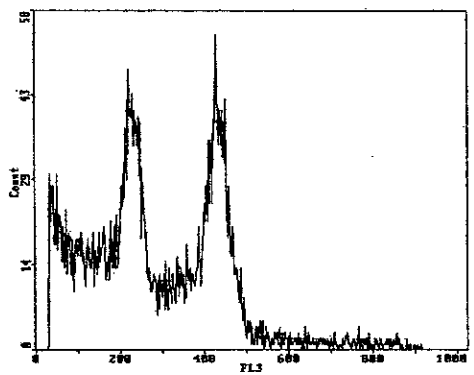
1mM Hydroxyurea 24 Hours + MM + 5μM MG132 for a further 24 Hours



50ng/ml Nocodazole 24 Hours



50ng/ml Nocodazole 24 Hours + MM for a further 24 Hours



50ng/ml Nocodazole 24 Hours + MM + 5μM MG132 for a further 24 Hours

Table 5.1. Cell Cycle Analysis of KIM-2 Cells Synchronised in G1/S Phase Prior to Treatment with Proteasome Inhibitor. KIM-2 cells were synchronised in G1/S phase and subsequently treated with 5μM MG132 as described in Figure 5.6b. Cells were harvested and fixed prior to staining with 5mg/ml Propidium Iodide. Results shown are the mean of three experiments, and are expressed as the percentage of cells which showed a Pre-G1 DNA peak, G1, S phase, or G2/M phase DNA profiles.

Table 5.2. Cell Cycle Analysis of KIM-2 Cells Synchronised in G2/Mitosis Prior to Proteasome Inhibition. KIM-2 cells were blocked in G2/Mitosis and subsequently treated with 5μM MG132 as described in Figure 5.6b. Cells were harvested, and fixed for flow cytometry analysis. 10^6 cells were stained with the nucleic acid stain Propidium Iodide. Results shown are means of three independent experiments, and data is shown as the proportion of cells showing a Pre-G1 DNA peak, G1, S phase, or G2/M phase DNA profile.

G1 Synch. + MG132	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)
0 Hours	4.7 (0.275)	69.6 (0.715)	10.2 (0.560)	10.7 (0.233)
2 Hours	8.8 (0.178)	56.0 (0.872)	12.2 (0.338)	13.3 (0.753)
4 Hours	11.9 (0.328)	58.3 (1.103)	13.2 (0.153)	7.1 (0.491)
6 Hours	14.4 (0.451)	55.3 (0.752)	16.2 (0.145)	5.3 (0.176)
8 Hours	16.6 (0.513)	53.1 (0.826)	16.4 (0.317)	7.3 (0.409)
10 Hours	19.1 (0.289)	50.6 (0.565)	15.7 (0.437)	7.1 (0.176)
12 Hours	30.9 (0.682)	33.5 (0.592)	21.1 (0.202)	8.0 (0.178)
14 Hours	34.9 (0.882)	31.5 (0.447)	23.1 (0.676)	4.4 (0.405)
16 Hours	35.3 (0.589)	27.9 (0.512)	26.1 (0.115)	5.9 (0.384)
18 Hours	40.6 (0.373)	25.4 (0.379)	21.6 (0.328)	4.8 (0.120)
20 Hours	42.1 (0.366)	19.0 (1.143)	24.5 (0.406)	5.6 (0.251)
22 Hours	44.0 (0.571)	21.5 (0.722)	25.0 (0.361)	2.9 (0.360)
24 Hours	40.5 (0.918)	23.8 (0.586)	25.3 (0.233)	2.0 (0.528)

G2 Synch. + MG132	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)
0 Hours	1.2 (0.187)	9.3 (0.315)	7.2 (0.088)	73.3 (0.529)
2 Hours	4.0 (0.226)	15.3 (0.283)	7.8 (0.145)	54.1 (0.731)
4 Hours	4.2 (0.311)	14.9 (0.342)	8.5 (0.260)	54.5 (0.484)
6 Hours	4.5 (0.265)	17.9 (0.256)	7.7 (0.273)	52.5 (0.375)
8 Hours	4.5 (0.377)	20.7 (0.314)	9.0 (0.404)	50.9 (0.384)
10 Hours	5.8 (0.447)	20.8 (0.531)	11.6 (0.251)	49.4 (0.435)
12 Hours	11.3 (0.553)	17.9 (0.784)	11.2 (0.448)	44.3 (0.405)
14 Hours	14.3 (0.722)	20.8 (0.569)	12.7 (0.215)	38.0 (0.617)
16 Hours	16.7 (0.871)	23.4 (0.617)	14.4 (0.375)	32.8 (0.721)
18 Hours	19.5 (0.673)	21.1 (0.332)	16.3 (0.470)	31.0 (0.762)
20 Hours	22.5 (0.455)	21.3 (0.474)	14.8 (0.669)	29.9 (0.811)
22 Hours	24.9 (0.524)	23.8 (0.514)	13.4 (1.117)	28.0 (0.589)
24 Hours	27.7 (0.379)	24.1 (0.616)	21.9 (0.346)	26.8 (0.437)

Table 5.3. Cell Cycle Analysis of KIM-2 Cells Synchronised in G1/S Phase Prior to Release into Maintenance Medium. KIM-2 cells were synchronised for 24 hours with 1mM Hydroxyurea. Following drug removal, cells were incubated in maintenance medium alone for the times shown. Cells were fixed and stained with Propidium Iodide for cell cycle analysis. Results detailed are the means of three experiments, and are presented at the proportion of cells in Pre-G1, G1, S phase, or in G2/M phase.

Table 5.4. Cell Cycle Analysis of KIM-2 Cells Synchronised in G2/ Mitosis Prior to Release into Maintenance Medium. Cells were synchronised in G2/Mitosis for 24 hours using 50ng/ml Nocodazole. Following drug removal, cells were incubated in maintenance media alone for the times shown. Cells were fixed and stained with Propidium Iodide for cell cycle analysis. Results shown are the mean of three experiments, and are presented as the proportion of cells in Pre-G1, G1, S Phase, or in G2/M phase.

G1 Synch. + MM	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)
0 Hours	13.2 (0.352)	67.9 (0.315)	14.2 (0.348)	7.4 (0.233)
2 Hours	7.1 (0.482)	70.4 (0.525)	10.2 (0.378)	7.9 (0.208)
4 Hours	7.2 (0.511)	63.2 (0.441)	18.7 (0.642)	6.4 (0.233)
6 Hours	6.8 (0.283)	53.2 (0.477)	17.8 (0.674)	16.5 (0.066)
8 Hours	12.6 (0.364)	48.6 (0.539)	19.2 (0.633)	12.6 (0.251)
10 Hours	13.6 (0.572)	42.6 (0.488)	26.7 (0.404)	10.6 (0.176)
12 Hours	13.6 (0.489)	33.6 (0.992)	35.9 (0.665)	13.6 (0.240)
14 Hours	23.8 (1.153)	34.0 (0.716)	26.3 (0.491)	10.1 (0.375)
16 Hours	18.2 (0.769)	41.6 (0.843)	23.9 (0.328)	8.9 (0.378)
18 Hours	22.4 (0.837)	41.7 (0.641)	20.9 (0.768)	7.2 (0.120)
20 Hours	22.4 (0.586)	46.9 (0.763)	18.5 (0.384)	8.9 (0153)
22 Hours	23.2 (0.618)	45.8 (0.568)	18.0 (0.550)	10.7 (0.264)
24 Hours	21.1 (0.459)	47.3 (0.761)	18.1 (0.296)	11.9 (0.208)

G2 Synch. + MM	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)
0 Hours	8.1 (0.158)	15.1 (0.281)	9.7 (0.260)	66.4 (0.519)
2 Hours	9.9 (0.413)	36.5 (0.457)	14.8 (0.503)	32.2 (0.923)
4 Hours	9.5 (0.356)	41.8 (0.378)	11.6 (0.218)	31.7 (0.895)
6 Hours	9.4 (0.245)	41.7 (0.672)	11.0 (0.328)	30.7 (0.321)
8 Hours	10.6 (0.359)	49.2 (0.525)	10.1 (0.378)	24.4 (0.578)
10 Hours	9.9 (0.418)	51.4 (0.871)	12.5 (0.352)	21.0 (0.721)
12 Hours	8.2 (0.238)	50.3 (0.762)	15.3 (0.173)	20.4 (0.204)
14 Hours	7.6 (0.279)	47.7 (0.414)	14.3 (0.703)	19.9 (0.608)
16 Hours	10.7 (0.562)	46.2 (0.337)	18.3 (0.438)	17.0 (0.536)
18 Hours	10.3 (0.518)	47.4 (0.179)	18.9 (0.433)	16.2 (0.260)
20 Hours	11.3 (0.316)	45.8 (0.456)	18.2 (0.776)	17.4 (0.173)
22 Hours	11.9 (0.541)	50.2 (0.913)	17.2 (0.883)	15.7 (0.233)
24 Hours	14.8 (0.447)	48.4 (0.785)	18.4 (0.497)	14.9 (0.273)

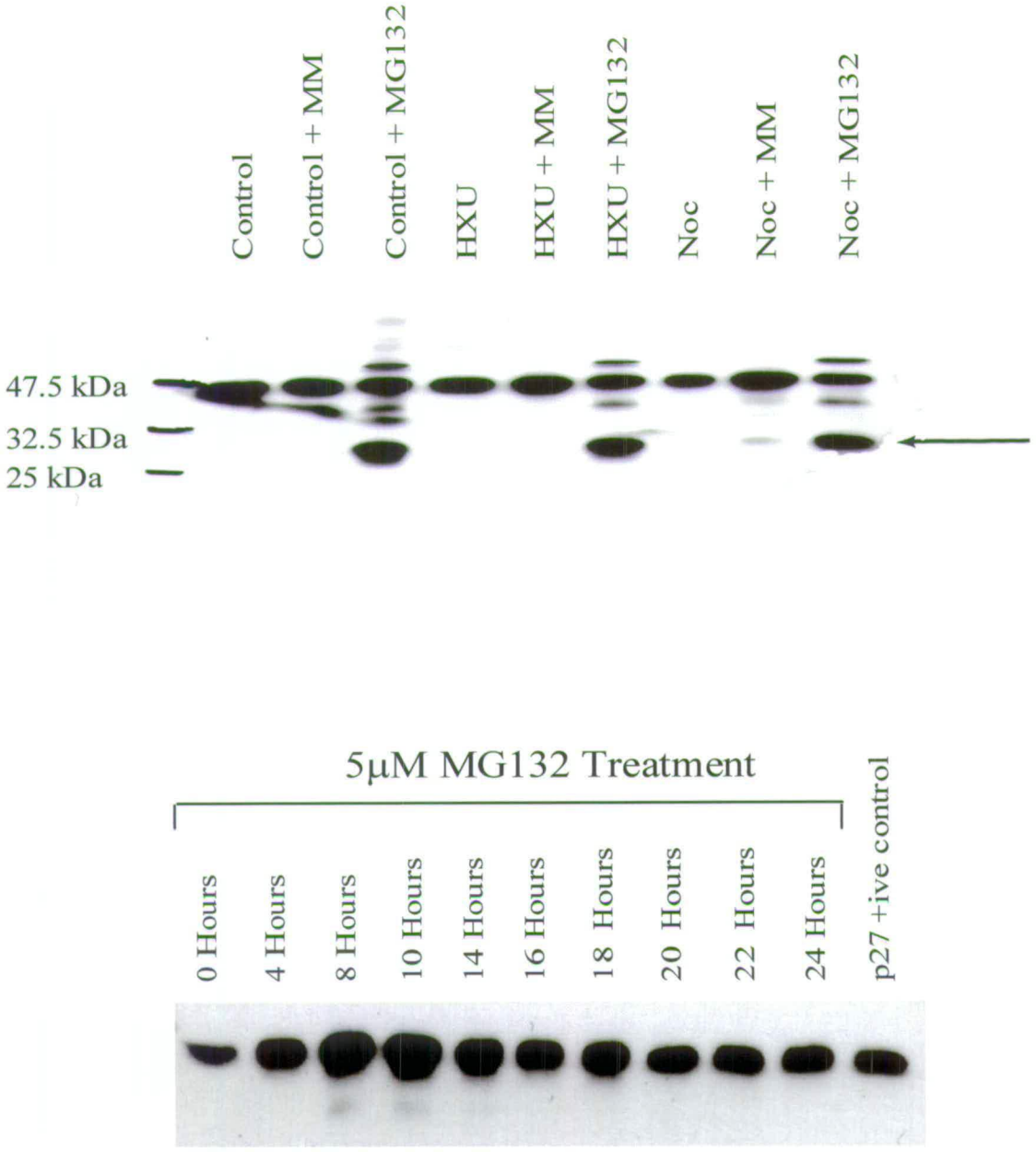
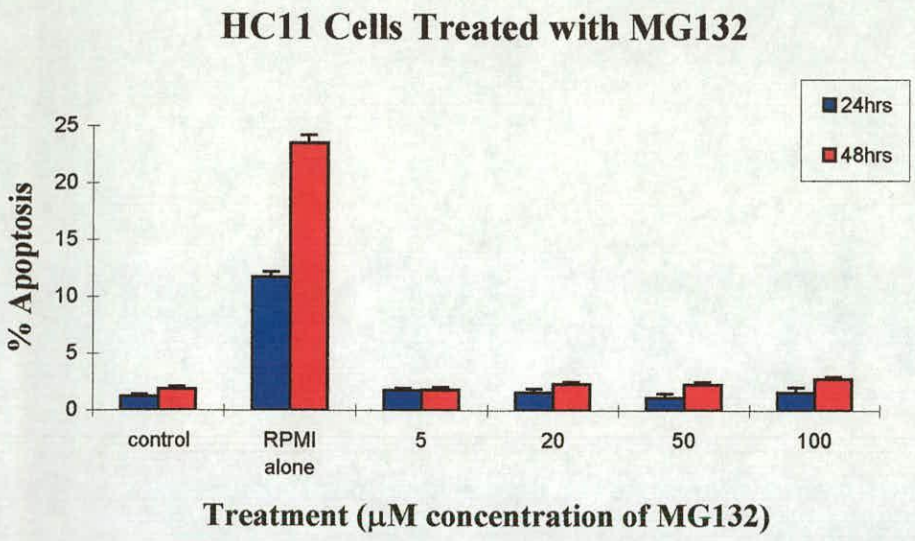


Figure 5.7 $p27^{Kip1}$ Expression in KIM-2 Cells treated with Proteasome Inhibitor. KIM-2 cells were treated with Hydroxyurea (HXU) at 1mM, Nocodazole (Noc) at 50ng/ml, or vehicle alone (control) for 24 hours. Cells were washed to remove the drug and subsequently treated with either maintainance media (MM) alone or MM supplemented with 5μM MG132 for an additional 24 hours (Upper panel). Conversely, KIM-2 cells were grown to confluence and treated over a 24 hour period with 5μM MG132 (Lower panel). Whole cell extracts were prepared and 10μg protein was subjected to SDS/PAGE analysis. This was followed by western blotting using a p27 monoclonal antibody (Transduction Laboratories). The p27 positive control was 5μg protein from HeLa cells stimulated with EGF (Transduction Laboratories). The lower panel represents a longer exposure to autoradiographic film that that shown in the upper panel.

(a)



(b)

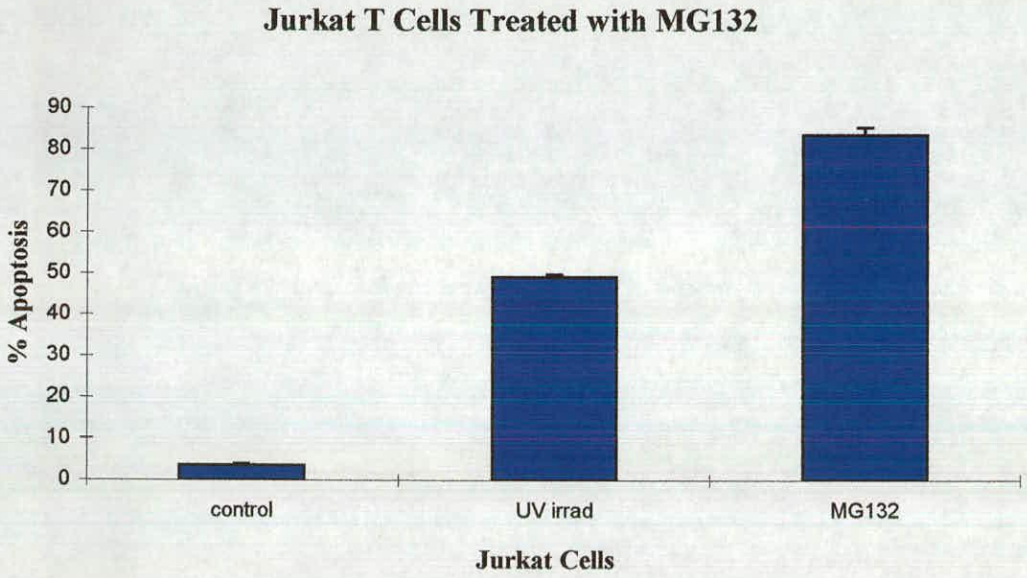


Figure 5.8 a and b Effect of Proteasome Inhibition on Mammary Epithelial Cells and Jurkat T Cells. (a) HC11 mammary epithelial cells were grown to confluence and treated with a range of concentrations of MG132 for 24 and 48 hours. Cells were harvested and fixed prior to staining with acridine orange. Cell death was observed morphologically by fluorescence microscopy and the data shown are the mean of three experiments ± sem, where 500 cells were counted for each sample presented. (b) Jurkat T cells were treated with either 2μM MG132 or 10mJ/cm2 of UV irradiation for 24 hours. Cells were harvested and fixed prior to staining with acridine orange. The results shown are the mean of three experiments ± sem, where 500 cells were counted for each sample presented.

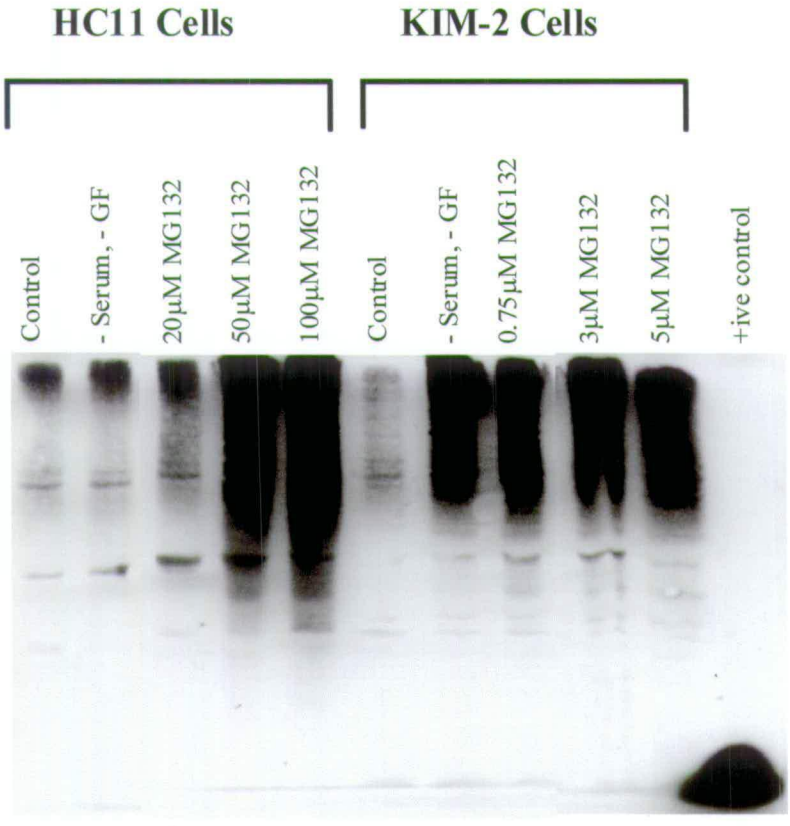


Figure 5.8c Accumulation of Ubiquitinated protein in Mammary Epithelial Cells following Proteasome Inhibition. KIM-2 and HC11 mammary epithelial cells were incubated with various concentrations of MG132 for 24 hours. Whole cell extracts were prepared and 10μg protein analysed by western blotting using a polyclonal ubiquitin antibody (DAKO). The +ive control sample is 5μg purified ubiquitin protein (Sigma). Additional controls for cell death are represented by cells that were serum and growth factor starved (-serum, -GF).

5.4 Induction of Apoptosis by Proteasome Inhibition is p53 Dependent

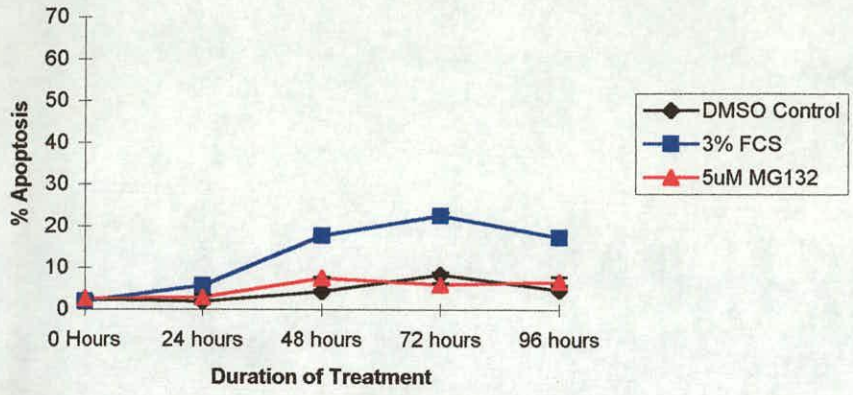
Many apoptotic pathways within the cell require the tumour suppresser protein p53. Results with HC11 cells suggested that functional p53 may be required for the induction of apoptosis by proteasome inhibition. To address this question in KIM-2 cells, we temperature switched the cells to 33°C to complex SV40 T Antigen (Tag) to p53. This can be achieved because KIM-2 cells were generated from transgenic mice carrying a temperature sensitive Tag driven by a milk protein gene promoter to direct expression to the mammary gland. At 33°C, Tag complexes with p53, the retinoblastoma gene product (Rb), and another Rb family member p107 resulting in a cell population with no functional p53 or Rb (Schreier and Gruber, 1990). This allows continual passaging of the cell line due to immortalisation. KIM-2 cells were routinely cultured at 37°C, a semi-permissive temperature because it was assumed that the Tag was partially active at this temperature. This was due to the observation that mice carrying high copy numbers of the transgene developed tumours (Gordon, 1997). In contrast to this, at 39°C the Tag is not functional due to a conformational change and subsequent degradation of this protein. This results in Tag being unavailable for binding to p53, Rb, and p107 and as a consequence the cells are no longer immortal. At 39°C, KIM-2 cells can only be grown for 3 to 4 days in culture before the cells spontaneously senesce. The experiment performed involved passaging cells and allowing them to grow at 37°C for 48 hours. The cells were then switched to 39°C for a full 24 hours before treatment with MG132 for an additional 24 hours. KIM-2 cells, which were switched to 33°C were passaged for 14 days prior to treatment. At 33°C and 37°C, MG132 treatment was continued for up to 96 hours and the cells analysed for apoptosis by Annexin V expression (Figure 5.9a). These results showed that cells at 33°C were resistant to apoptosis by proteasome inhibition. Serum and growth factor starvation was included as a control in this group of treatments. This controlled for the slower onset of death at 33°C. The delay in onset of death was approximately 48 hours and this treatment induced 15-20% apoptosis regardless of the temperature. At 37°C, KIM-2 cells are immortal due to "leaky" expression of Tag, which would suggest that some of the p53 is

complexed to Tag at this temperature. If this is the case then we should expect to see enhanced death by proteasome inhibition at 39°C. This hypothesis was confirmed, only 10% non-apoptotic cells (unstained with either propidium iodide or Annexin V), as determined by Annexin V assay, were remaining in the culture following a 24 hour treatment period at 39°C. This compared to approximately 65% non-apoptotic cells observed at 37°C. These results further suggest a role for functional p53 in the apoptotic response induced by proteasome inhibition. To determine whether KIM-2 cells cultured at 33°C still responded like mammary epithelial cells, we induced these cells to differentiate with lactogenic hormones. Figure 5.9b shows β-casein expression in KIM-2 cells cultured at 37°C over a 12 day period. Cells at 33°C were differentiated for a full 12 days. This result confirmed that cells cultured at 33°C were still prolactin and dexamethasone responsive. When we compared the level of β-casein expression it was obvious that cells at 33°C did not express as much of this milk protein as the equivalent cells at 37°C. β-casein expression in 12 day differentiated cells at 33°C was equivalent to 4 days differentiation at 37°C. These results suggest that KIM-2 cells at 33°C were still responding to prolactin like mammary epithelial cells, although they were no longer as responsive as their counterparts at 37°C.

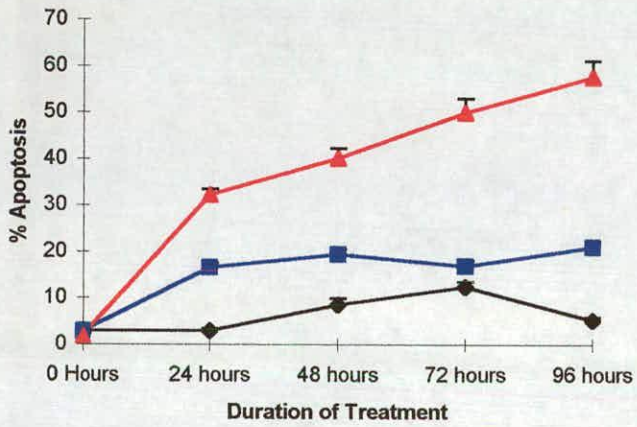
Overexpression of p53 is sufficient to induce apoptosis in response to a range of insults (Evan *et al.*, 1995). It was assumed that p53 levels may have been elevated in KIM-2 cells following proteasome inhibition as this protein has also been found to be a substrate of the 26S proteasome (Scheffner *et al.*, 1990). An elevation in p53 protein alone could represent the apoptotic signal following MG132 treatment. We therefore examined the level of p53 protein in cells treated with MG132. At 24 hours the levels of p53 protein are similar in control cells and cells treated with MG132, suggesting that there is no long term accumulation of p53 in these cells (Figure 5.10). The p53 expression over a 24 hour period of MG132 treatment was also seen to remain constant suggesting that KIM-2 mammary epithelial cells are not undergoing apoptosis as a consequence of the perturbation of p53 expression.

Figure 5.9a. Comparison of Apoptosis induced by proteasome inhibition in KIM-2 cells grown at 33°C, 37°C, and 39°C. KIM-2 cells were cultured at 33°C for 14 days prior to treatment, and at 39°C for 48 hours prior to treatment. Cells were treated with vehicle alone (DMSO), serum and growth factor starved (3% FCS), or with 5μM MG132 over 24 hour period for a maximum of 96 hours. Death was initially assayed over a 72 hour period. The 96 hour timepoints represent any additional apoptosis after 72 hours as medium was replaced on the cells at this stage. Cells were harvested and 10^5 cells were analysed for cell death using an Annexin V assay. The data shown are a representative of the mean of three independent experiments, and are expressed as the percentage of Annexin V positive cells ± sem.

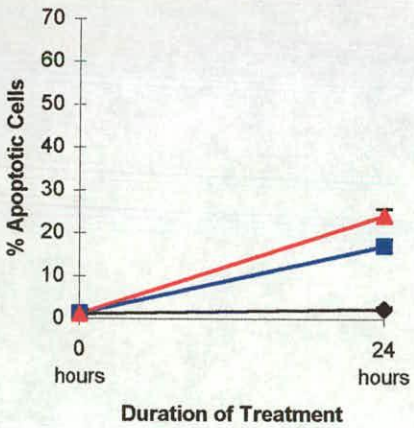
KIM-2 Cells 33°C



KIM-2 Cells 37°C



KIM-2 Cells 39°C



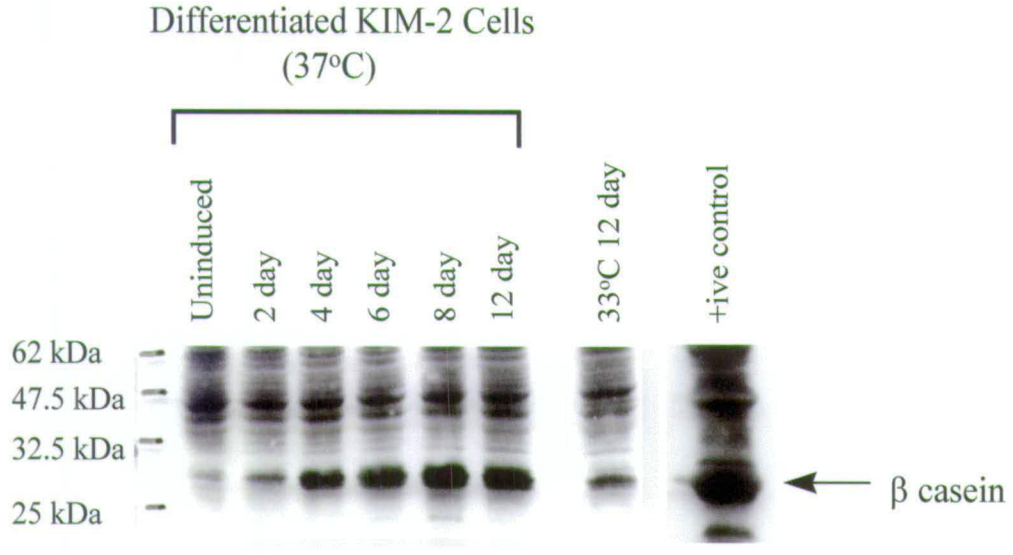
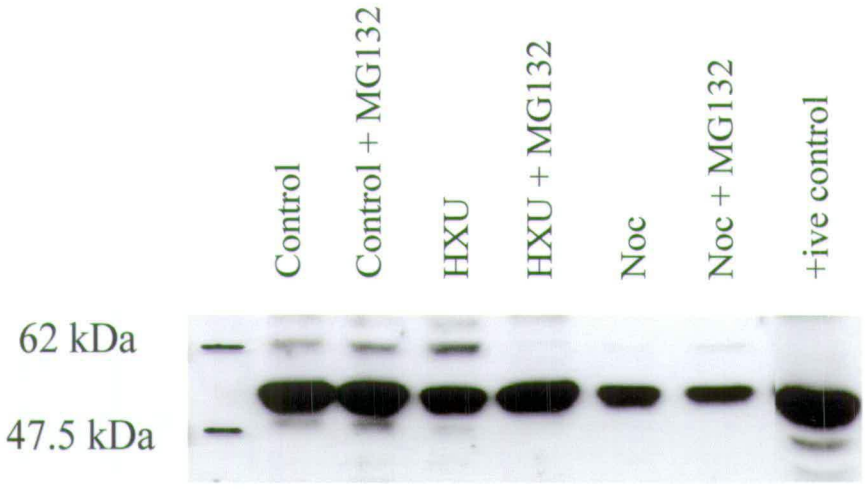


Figure 5.9b β-casein Expression in KIM-2 Cells Cultured at 33°C. KIM-2 cells were differentiated at 37°C for up to 12 days in the presence of dexamethasone and prolactin. KIM-2 cells transferred to 33°C were cultured at this temperature for at least 14 days prior to the induction of differentiation. Whole cell extracts were prepared and 10μg protein subjected to SDS/PAGE analysis. Western blot analysis followed using a murine polyclonal β-casein antibody (kindly provided by Dr. B Binas). The uninduced sample represents KIM-2 cells which were grown to confluence but did not receive a differentiation stimulus. The +ive control sample represent 10μg protein extracted from the mammary gland of a mouse at day 2 lactation.



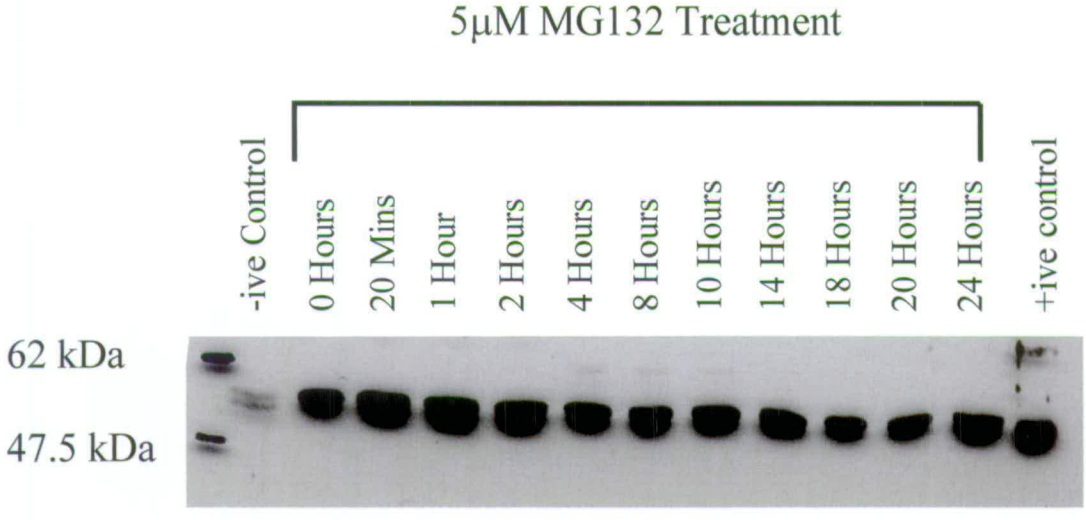


Figure 5.10 Effect of Proteasome Inhibition on p53 levels in KIM-2 cells. The upper panel represents KIM-2 cells that were treated with either Hydoxyurea (HXU) at 1mM, Nocodazole (Noc) at 50ng/ml, or vehicle alone (control) for 24 hours. Cells were washed to remove the drug and subsequently treated with 5μM MG132 for an additional 24 hours. The lower panel represents cells that were grown to confluence and treated for up to 24 hours with 5μM MG132. Whole cell extracts were prepared and 10μg protein was subjected to SDS/PAGE analysis. Western blotting following using a polyclonal p53 antibody (CM5 clone). The –ive control is differentiated KIM-2 cells, which express less p53 as these cells are not actively progressing the cell cycle. The +ive control samples are KIM-2 cells treated with 10mJ/cm^2 of UV irradiation.

To test the hypothesis that p53 was essential for apoptosis induced by proteasome inhibition more empirically, we obtained ES cell lines which contained either wild type p53 (E14 cells), or were null for p53 (R72D27 cells). One p53 allele had been deleted by gene targeting in these ES cells and the second allele spontaneously gained a mutation resulting in no functional p53 in this cell population (Corbet, 1998). These ES cells were treated with 3 different concentrations of MG132 for up to 48 hours and assayed for cell death and cell cycle status. The levels of apoptosis observed were greatly enhanced in the p53 wt cells as compared to the p53 null cells (Figure 5.11a). Cell cycle analysis identified a G2/mitosis block in both cell types in contrast to death which was observed only in the p53wt cells. At higher concentrations this cell cycle blockage was accompanied by the appearance of a pre-G1 DNA peak in the p53 wt cells e.g. 12.5% in the control (DMSO) cells, which increased to 65.2% with 10μM MG132 treatment (Figure 5.11b). The appearance of a pre-G1 peak in flow cytometry correlates with the appearance of apoptotic cells (Compton *et al.*, 1988). In contrast the p53 null cells remained blocked in G2/mitosis with very little death observed e.g. 16.1% pre G1 staining cells for 10μM MG132 treatment (Tables 5.5 and 5.6). These results are consistent with a role for p53 involvement in apoptosis induced by proteasome inhibition.

5.5 Discussion

The results in this chapter demonstrate that compounds capable of interfering with proteasome function induce apoptosis. KIM-2 mammary epithelial cells were incubated with inhibitors of the chymotrypsin-like function of the proteasome as well as with unrelated protease inhibitors. Of the two inhibitors that were capable of inducing cell death in these cells, both have been attributed with perturbing the 26S proteasome and its degradation pathway. Both these inhibitors have similar

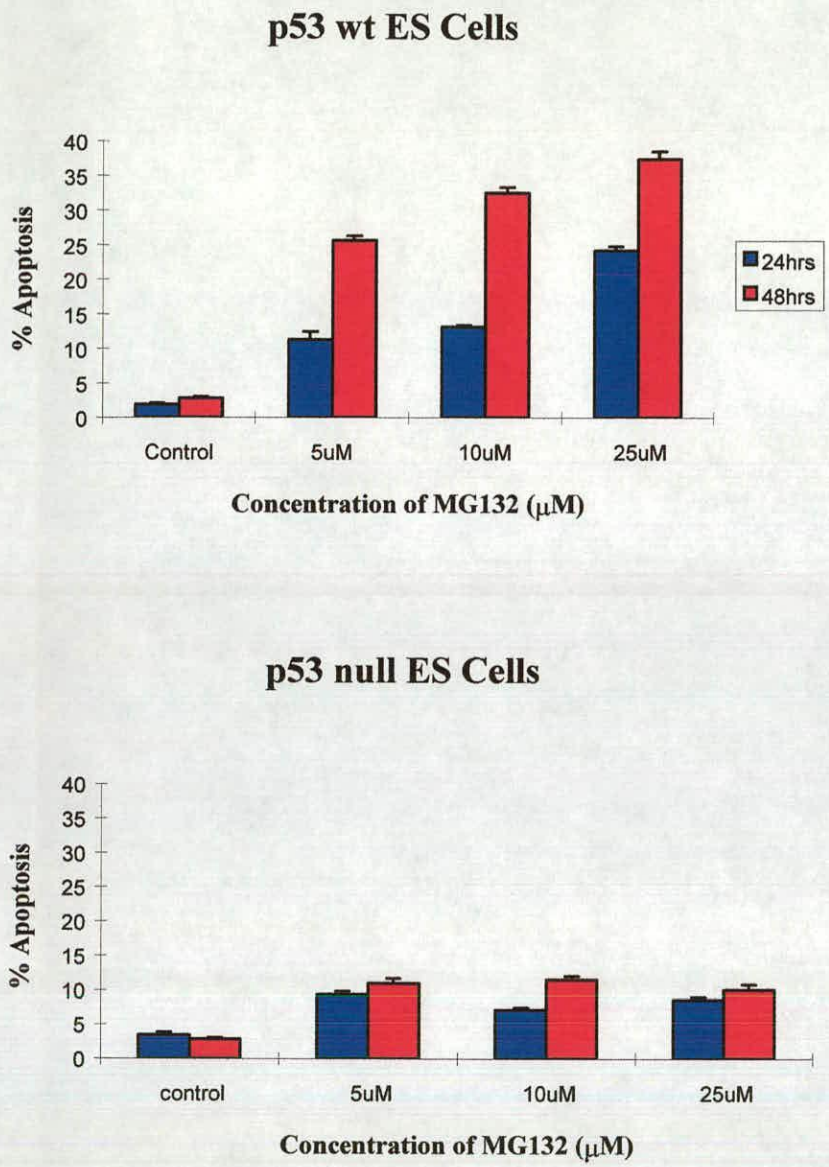
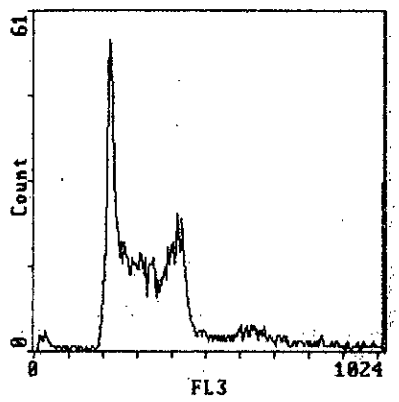
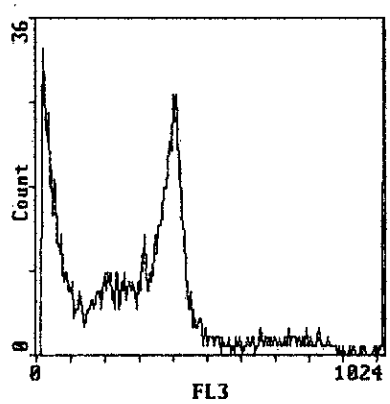


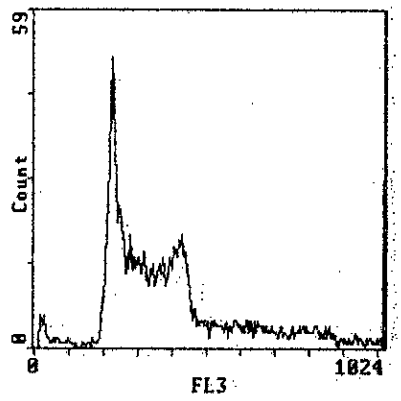
Figure 5.11a p53 Dependence on Apoptosis Induced by Proteasome Inhibition. ES cells containing either wild type (wt) p53 (E14 cells) or which were null for p53 (R72D27 cells) were grown to confluence and treated with different concentrations of MG132 for up to 48 hours. Cells were harvested and an Annexin V assay performed. The results shown are expressed as the percentage of Annexin V positive cells, and represent the mean of three experiments ± sem.



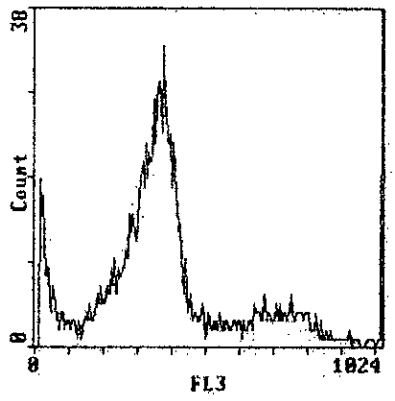
p53 wt Control



p53 wt 10μM MG132



p53 null Control



p53 null10μM MG132

Figure 5.11b Effect of p53 on Cell Cycle distribution of ES Cells treated with Proteasome Inhibitor. ES cells which were wild type (wt) or null for p53 were cultured in full maintenance medium alone or supplemented with 10μM MG132 for 24 hours. Cells were harvested and fixed prior to staining with propidium iodide. Cell cycle analysis was performed using a Coulter Epics flow cytometer.

Table 5.5. Cell Cycle Analysis of p53 wt ES Cells Treated by Proteasome Inhibition. Cells were treated with three concentrations of MG132 for 24 and 48 hours. Cells were subsequently fixed and stained with PI for cell cycle analysis. Results shown are the mean of three experiments, and are expressed as the proportion of cells showing a Pre-G1 DNA peak, G1, S phase, or G2/M phase DNA profile.

Table 5.6. Cell Cycle Analysis of p53 null ES Cells Treated by Proteasome Inhibition. Cells were treated with three concentrations of MG132 for 24 and 48 hours. Cells were subsequently fixed and stained with PI for cell cycle analysis. Results shown are the mean of three experiments, and are expressed as the proportion of cells showing a Pre-G1 DNA peak, G1, S phase, or G2/M phase DNA profile. UnQ represents samples that were unable to be quantified. The cells in these samples accumulated in G2/M phase and this peak displayed a shoulder that obscured the proportion of cells in G1 and S phase. The proportion of cells at any stage further than G1 therefore could not be precisely quantified.

p53wt Cells	24 Hours				48 Hours			
	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)
Control	2.4 (0.176)	30.1 (0.573)	36.0 (0.737)	27.2 (0.664)	12.5 (0.385)	35.8 (0.754)	22.0 (0.866)	20.7 (0.529)
5μM MG132	11.8 (0.452)	10.8 (0.241)	15.1 (0.608)	53.2 (1.105)	36.8 (0.719)	6.4 (0.299)	8.2 (0.763)	39.3 (0.832)
10μM MG132	30.9 (0.682)	10.1 (0.431)	20.5 (0.589)	29.7 (0.754)	65.2 (1.153)	6.2 (0.317)	6.1 (0.360)	14.6 (0.744)
25μM MG132	45.2 (0.815)	13.5 (0.693)	18.5 (0.523)	6.8 (0.354)	77.8 (0.128)	4.4 (0.526)	4.9 (0.510)	6.9 (0.371)

p53null Cells	24 Hours				48 Hours			
	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)	Pre-G1 (± sem)	G1 (± sem)	S phase (± sem)	G2/M (± sem)
Control	3.7 (0.238)	28.5 (0.383)	27.5 (0.584)	28.5 (0.757)	6.7 (0.335)	32.7 (0.664)	UnQ †	UnQ †
5μM MG132	15.6 (0.412)	11.3 (0.197)	11.5 (0.623)	49.7 (0.548)	14.8 (0.574)	10.7 (0.458)	UnQ †	UnQ †
10μM MG132	12.8 (0.395)	12.9 (0.511)	3.8 (0.417)	75.2 (0.898)	16.1 (0.592)	10.1 (0.286)	UnQ †	UnQ †
25μM MG132	15.5 (0.452)	8.6 (0.587)	10.6 (0.504)	65.9 (0.995)	16.8 (0.411)	9.7 (0.428)	UnQ †	UnQ †

biochemical inhibitory effects on calpains but MG132 is three times more effective at inhibiting the 26S proteasome than ALLM (Rock *et al.*, 1994) suggesting that the difference in response observed between these inhibitors in this study is due to proteasome inhibition and not to calpain or cathepsin B inhibition. Given that MG132 had no effect on KIM-2 cells at 33°C, then the effects observed at 37°C and 39°C cannot to attributed to non-specific cytotoxicity. Therefore it would seem highly probably that the ubiquitin-proteasome pathway of protein degradation is linked to the regulation of apoptotic cell death.

A major function of the 26S proteasome is the degradation of cell cycle proteins e.g. it is responsible for the degradation of cyclins, cdks, cdk inhibitors, c-Myc, p53, Rb, and E2F-1. All of the above proteins are involved in cell cycle regulation and have been reported to be actively degraded at specific points during the cell cycle. In some cases, their degradation is necessary to ensure correct progression through the cell cycle (Nurse, 1994). Proteolysis is involved in controlling two stages in the cell cycle: the G1/S phase transition through the degradation of cdc34, and mitosis initiation and progression through the degradation of mitosis promoting factor (MPF). The effects exerted on the cdc34 pathway have shown that ubiquitination of the substrates is controlled by modulation of susceptibility of the substrate, rather than regulation of ubiquitinating enzymes as is generally the case for this degradation machinery (King *et al.*, 1996). The irreversibility of proteolysis is exploited by the cell to provide directionality to the critical steps in the cell cycle. Proteolysis also plays a prime role in regulating the timing of the cell cycle transitions, suggesting that the interdependence of CDK-activity and ubiquitin-dependent proteolysis ensure that cell cycle events occur in the proper order. Therefore it seems plausible that if this pathway is perturbed then the major effects seen will be on the progression of the cell cycle. Our results agree with previous reports showing that in actively proliferating cells, proteasome function is essential for cell cycle progression (Drexler, 1997, Lopes *et al.*, 1997). In this study we observed a limited amount of death in differentiated mammary epithelial cells. This would suggest that disruption of the 26S proteasome in a non-proliferating cell may result in an entirely different outcome than that observed in a dividing cell.

Evidence to support this theory has come from studies in Rat1 cells, which had been rendered quiescent by serum starvation (Lopes *et al.*, 1997). Proteasome inhibition failed to induce apoptosis in these quiescent cells. In contrast, both proliferating and differentiated PC12 cells could be induced to undergo apoptosis. Data conflicting with these results comes from work in neurons (Sadoul *et al.*, 1996) and thymocytes (Grimm *et al.*, 1996), which were seen to require proteasome function for induction of an apoptotic response. These results can be explained by the non-proliferating status of these two cell types. Indeed Grimm and colleagues (1996) have shown that in thymocytes, long term exposure to proteasome inhibitors can induce a background level of cell death. Both the thymocyte and neuron experiments suggested that the proteasome acted upstream in the apoptotic response prior to the activation of the caspases. One set of experiments that should answer the question of the relevance of the cell cycle on the apoptotic response is to render the MEC quiescent. Attempts to block these cells and treat with MG132 failed due to the inability of the drugs used to completely synchronise the cell population. Ideally, a drug which blocks cells at a specific stage of the cell cycle and has low toxicity to the cells should resolve this difficulty. Taken together, the data available would implicate the proteasome in functioning in both cell survival and cell death pathways. Whichever pathway the cell executes may depend on its proliferative state and other cell specific factors, which remain unknown to date.

The results presented described a dependence on p53 function for the apoptotic response to proteasome inhibition. KIM-2 cells containing SV40 Tag at the non-permissive of 39°C underwent >80% apoptosis in response to proteasome inhibition, whereas at the permissive temperature of 33°C no induction of death was observed. Results from p53 null ES cells and HC11 cells confirmed this result. The results observed in HC11 cells can be explained due to a mutation in p53 in this cell line (Merlo *et al.*, 1995), providing further evidence in favour of a role for p53 function in this pathway. KIM-2 MEC treated with proteasome inhibitor were observed to accumulate in G1/S phase prior to undergoing apoptosis. These cells also had difficulty traversing G2/M phase efficiently. In contrast to this ES cells arrested in G2/M phase prior to the induction of apoptosis. The evidence for cell

cycle arrest by proteasome inhibition is also conflicting. HeLa cells arrest in G2/M (Wojcik *et al.*, 1996), identical to CHO cells containing an ubiquitin activating enzyme mutation (Kulka *et al.*, 1988). Yeast cells containing ubiquitin mutants also G2/M arrest (Finley *et al.*, 1994), whereas the human leukemic HL60 cells arrest in G1/S phase (Drexler, 1997). It is possible that a G1/S phase arrest could be induced as a result of a p53 dependent apoptotic response. p53 is linked to the G1/S phase checkpoint through a DNA damage pathway, but p53 is also documented to trigger apoptosis via other responses e.g. in response to metabolite imbalance or to calcium phosphate (Evan *et al.*, 1995) and can also induce death from other stages of the cell cycle e.g. from G2/M following DNA damage induced apoptosis (Guillouf *et al.*, 1995).

The difference in cell cycle arrest cannot be explained by the presence of Tag itself. SV40 Tag complexes with p53, Rb, and p107 at the permissive temperature of 33°C. p53 is a known target of the ubiquitin-proteasome pathway. Previous studies have shown that the human papilloma virus (HPV) E6 protein, in association with a cellular factor named E6 associated protein (E6AP), complexes with p53 and promotes its degradation. E6AP functions as an E3 or ubiquitin ligase in this reaction; it catalyses the transfer of ubiquitin from the ubiquitin conjugating enzyme to the p53 protein. This facilitates the degradation of p53 by the 26S proteasome. Until recently, it was unknown whether E6AP acted to regulate p53 turnover in the absence of HPV infection in the cell. Maki and colleagues (1996) have recently identified p53-ubiquitin conjugates in cells treated with proteasome inhibitor that suggested that *in vivo* in the absence of viral infection, p53 was degraded by the 26S proteasome. The search for the *in vivo* equivalent of E6AP was resolved by the observation that the oncoprotein mdm2 interacted with p53. When p53 is bound by mdm2, its cell cycle arrest and apoptotic functions are inhibited (Kubbutat *et al.*, 1997). This interaction with p53 results in a large reduction of p53 protein levels through enhanced proteasome-dependent degradation. Mdm2 function has been observed to be crucial for regulating p53 function from studies with transgenic mice. Mice with mdm2 gene deletions show lethality early in embryogenesis only when the animals carry a functional p53. Mice with deletions of both mdm2 and p53 develop

normally (Montes de Oca *et al.*, 1995, and Jones *et al.*, 1995). Other evidence for mdm2 control of p53 function comes from studies of human sarcomas, where amplification of the mdm2 gene is found at high frequencies in tumours containing unaltered p53 (Oliner *et al.*, 1992). This observation is consistent with the theory that high levels of mdm2 protein are as effective as a p53 mutation in knocking out the p53 pathway. The N-terminal region of mdm2 was shown to be essential for p53 inhibition, and the remainder of the protein is thought to have other functions, which may include interactions with Rb (Xiao *et al.*, 1995) and E2F (Martin *et al.*, 1995). Although mdm2 binds to p53 and masks its transactivation domain, it is also transcriptionally activated by p53, thereby acting in an autoregulatory manner. This may contribute to the maintenance of low p53 levels in normal cells. Haupt and co-workers (1997) have shown that elevated levels of mdm2 can repress mutant p53 accumulation. This work suggested that in tumour derived cells, mdm2 acts to degrade p53 in situations where p53 would be otherwise stabilised. This may be a function of mdm2 inhibiting the phosphorylation of p53 on N-terminal sites and thus acting to hinder stress induced p53 stabilisation. The data on p53 mutations currently conflicts with what is known regarding p53 degradation. Most of the mutations identified to date appear to result in an increase in the half-life of the protein (Brown *et al.*, 1997) although they are clustered in areas of the protein which are not expected to alter ubiquitin-mediated degradation. The reasons for this stabilisation are not understood at present although p53 PEST sequences are often conserved in p53 mutants suggesting that there should be no effect on stability. PEST sequences act as one of the signals that target a protein for degradation by the 26S proteasome pathway. The precise signals which determine whether p53 is degraded by the proteasome are not completely understood at present, but may be affected by p53 phosphorylation state, PEST sequences at the N-terminus, and also by p53 conformation.

The results presented in this chapter suggested that p53 is not degraded by the 26S proteasome in KIM-2 MEC. This data conflicts with what it currently known about p53 degradation. It has been shown that in PC12 and Rat 1 cells, a dominant negative p53 could abrogate the apoptotic response seen as a consequence of

proteasome inhibition (Lopes *et al.*, 1997). In cells expressing wild type p53, proteasome inhibition resulted in apoptosis and an upregulation of the p53 target genes mdm2 and p21. Scheffner and colleagues (1990) observed that in HPV infected cells, the E6 stimulated degradation of p53 was not inhibited by SV40 Tag, suggesting that E6 and Tag may bind to different regions of p53 or that E6 has a higher affinity for p53 than does Tag. These results would suggest that p53, when complexed to Tag appears to be accessible to the degradation machinery of the 26S proteasome. If it is proposed that mdm2 binds to an identical region on p53 as E6, the presence of Tag cannot explain the difference in p53 response in these cells. Further evidence suggesting that the p53 response in cells at 37°C is normal is provided by the observation that KIM-2 cells will undergo >75% apoptosis as a response to UV irradiation. The experiments carried out with the temperature switched KIM-2 cells and those with ES cells suggest that the response we observed was p53 dependent. KIM-2 cells do not appear to be undergoing apoptosis as a response to elevated levels of p53 protein. These observations would suggest that part of the response to inhibition of the proteasome is cell type specific. The difference in p53 response between mammary cells and neuronal cells may be a consequence of Tag complexing to key cell cycle regulatory proteins, which themselves are targets of this degradation pathway. It is equally plausible that the presence of SV40 Tag may alter the susceptibility of p53 to this particular proteolytic complex. An additional explanation exists for the stability of p53 in KIM-2 cells. It has been shown in our laboratory that all of the p53 in these cells is complexed to Tag at 33°C and that at the semi-permissive temperature of 37°C only a proportion of the p53 present in the cell is bound by Tag. As the stability of p53 in this study was examined in cells grown at 37°C, it is possible that the p53 complexed to Tag was stabilised whilst the free p53 was available to the degradation machinery. This may have resulted in the net effect of no observed changes in p53 levels in these cells due to the presence of more than one pool of intracellular p53. Further experiments examining the stability of p53 at 33°C and 39°C are required to address this possibility.

In KIM-2 cells, Rb function is also abrogated by SV40 Tag. Rb when

complexed to Tag is unable to function in regulating the activity of E2F-1. E2F proteins are a family of transcription factors composed of 5 E2F proteins (1-5) and three DP family members (1-3). E2Fs and DPs form heterodimers, which bind and transactivate promoters of several genes whose products are active in cell cycle regulation or DNA replication (Hoffman *et al.*, 1996). E2F proteins bind hypophosphorylated Rb (pocket) proteins to stabilise themselves. During the G1 stage of the cell cycle Rb binds in a hypophsophorylated form to E2F-1 suppressing its transactivation activity thus converting it to a transrepressor. In late G1, cdks act to phosphorylate Rb leading to a dissociation of the Rb/E2F-1 complex. This allows E2F-1/DP heterodimers, in conjunction with a cdk3 kinase activity, to reach their maximal transactivation potential (Hoffman and Livingston, 1996). After peaking at G1/S and early in S phase, the transactivation activity of E2F-1 decreases in a cyclin A/cdk2 dependent manner. This is thought to act to decrease the DNA binding capacity of E2F-1 (Krek *et al.*, 1994). Another level of control is exerted on this pathway with the degradation of E2F family proteins by the 26S proteasome. The C-terminal region of E2F-1 was seen to be essential for its degradation. It is thought that Rb acts as an inhibitor of E2F-1 degradation by steric hinderance. The epitope within E2F-1 that confers its ubiquitination and degradation by the 26S proteasome lies in close proximity to its pocket protein binding domain (Hateboer *et al.*, 1996). This interaction with Rb prevents E2F-1 degradation by the 26S proteasome by masking its degradation signal, and thus the protein is stable at the point in the cell cycle, G1/S phase transition, where its activity is essential for regulation (Hoffman *et al.*, 1996). The same is true for the regulation of activity of E2F-4 and p107 complexes. As a consequence proteasome inhibition may result in elevated levels of E2F-1, which have been reported to promote S phase entry followed by apoptosis (Shan and Lee, 1994). This suggests that death would be induced from G1/S phase of the cell cycle, a similar effect to that which was observed in KIM-2 cells treated with proteasome inhibitor. The presence of viral proteins e.g. Adenovirus E1 proteins (E1A) results in dissociation of Rb/E2F-1 complexes. This action was thought to be counterproductive to the cell as it was expected to unmask E2F-1's degradation signal, but E1A was observed to act in stabilising E2F activity and preventing its turnover (Campanero and Flemington, 1997). The mode of action of

this stabilisation is unclear at present but is obviously how these viral proteins act to transform a cell without inducing apoptosis. Whether Tag can act in the same manner as E1A to stabilise E2F-1's activity is unknown at present. In KIM-2 cells Tag and proteasome inhibition might act in concert to result in an overexpression and/or stabilisation of E2F proteins. It is possible that the co-operation of these two independent pathways to induce elevated E2F proteins can act to induce apoptosis.

In KIM-2 cells elevated levels of the G1 restriction point protein, p27 were observed. The observation of an increase in p27 expression is attributed to the inhibition of the proteasome as p27 has not been described as being processed by any other proteases (Vlach *et al.*, 1997). Previous work has shown that overexpression of any one of the cdk inhibitors can result in a G1 arrest (Vlach *et al.*, 1996). As p27 levels were observed to be elevated in KIM-2 cells following proteasome inhibition it is possible that this may contribute to the G!/S phase arrest observed prior to apoptosis. Indeed, overexpression of any number of cell cycle proteins can induce arrest and apoptosis e.g. a non-degradable mutant cyclin A expressed in the mammary gland of transgenic mice resulted in increased apoptosis (Bortner *et al.*, 1995). p27 associates with and inhibits cyclin E/cdk2. This induces an accumulation of Rb and p130 in their hypophosphorylated forms and also arrests cells in G1. The expression of c-*myc* prior to p27 results in inactivation of the cyclin E/cdk2 complex and allows continuous cell proliferation in the absence of p27. p27 and c-*myc* have opposite roles in growth control in response to mitogens e.g. p27 is induced by serum deprivation and is required for G1 arrest (Coates *et al.*, 1996), whereas withdrawal of mitogens in the presence of c-*myc* prevents cell cycle arrest (Evan *et al.*, 1992). Activation of c-*myc* is also seen to induce p27 degradation (Rudolph *et al.*, 1996). Vlach and co-workers (1997) have shown that degradation of p27 is dependent on its association with active cyclin E/cdk2, and also that p27 must be phosphorylated on its conserved cdk2 binding motif in order to be degraded by the proteasome. C-Myc is also a known substrate of the ubiquitin-proteasome pathway. It would be of interest to examine the levels of c-Myc protein in these cells as c-Myc expression is generally low when p27 levels are elevated. This would confirm the theory that a cell is undergoing apoptosis through conflicting signals. Furthermore the proteasome

is implicated in functioning during S phase. Ubiquitination of histones have been observed during S phase, which is an essential function for the unwinding and replication of the DNA, although no degradation of ubiquitinated histones has been described (Vassilev *et al.*, 1995). These observations would further suggest an inability for a cell deficient in proteasome function to traverse the G1/S phase of the cell cycle.

The mechanism of apoptotic cell death induced by proteasome inhibition is assumed to be highly complex. The 26S proteasome is documented to degrade a wide range of proteins including proteins involved in the control of the cell cycle. A major consequence of proteasome inhibition is elevated levels of cell cycle proteins as well as the induction of an apoptotic response. This inhibition has an outcome leading to inappropriate expression of control proteins for a particular stage of the cell cycle. Alone, this one event can act to result in the induction of apoptosis. By disruption of this degradation pathway a number of cell cycle proteins will be inappropriately expressed. The outcome of this is similar to above where a cell may also be induced towards an apoptotic response, but as a result of conflicting signals. Therefore death from proteasome inhibition may occur due to elevated levels of one protein whose expression levels govern cell survival, or as a consequence of conflicting signals given to a cell from a number of incorrectly expressed proteins. For example, p53 is known to be degraded by the proteasome, therefore elevated levels may be due directly to inhibition or may be part of a p53 dependent apoptotic response to aberrant expression of another protein which itself induced the apoptotic response. Determination of the effector mechanism of this apoptotic response requires the removal of each substrate individually to resolve if only one signal or a number of signals are sufficient to induce cell death. At present the dissection of the precise signal that induces apoptosis as a result of proteasome inhibition is unknown. What has become clear recently is that the 26S proteasome degradation pathway acts to regulate key control mechanisms of the cell cycle.

An apoptotic response and progression through the cell cycle initially appear as two unrelated processes with distinct biological endpoints. However, it has

become more apparent that the processes of cell loss and cell gain must be balanced, which suggested that these two antagonistic processes were linked. The evidence supporting this notion was based on the observations that some of the morphological changes occurring during apoptosis are reminiscent of mitosis e.g. cells lose their attachments, chromatin becomes condensed and nuclear laminins are phosphorylated and disassemble. The processes of apoptosis and mitosis are of similar duration, each taking around 30-60 minutes to complete (Ucker, 1991). Further evidence supporting this connection was based on the observation that healthy cells undergo apoptosis as part of their normal process of development suggesting that cell death was a normal physiological process. Neoplastic lesions that generate uncontrolled cell proliferation have also been seen to act as potent triggers of apoptosis providing further evidence of a link between these two pathways (Evan *et al.*, 1995). Evidence for an interdependence between these two processes is strong. It is further supported by the observation that a number of proteins that act to control cell cycle progression also induce apoptosis e.g. E1A, p53, Tag, and c-*myc*. The difference in response induced is often dictated by the level of the protein expressed e.g. elevated levels of c-*myc* can induce apoptosis. Whether the same biochemical pathways induce both of these responses is unknown at present. Given that a change in proteins levels is often all that is required for a switch from proliferation to an apoptotic response, then the 26S proteasome must be a prime candidate for control of these processes. The proteasome is known to be involved in degrading key regulatory proteins implicated in cell cycle control and has also been observed to be essential for an apoptotic response. Whether this is through the degradation of the same set of substrates that act to induce survival under one set of circumstances and death under another has yet to be resolved.

Chapter 6:

Analysis of the Effects of a Dominant Negative TBP1 Variant On KIM-2 Mammary Epithelial Cells

6.1 Introduction

In chapter 5 it was demonstrated that inhibition of the 26S proteasome using a peptide aldehyde inhibitor in KIM-2 MEC resulted in the induction of apoptosis. The apoptosis observed was shown to be a p53-dependent response, and the cells arrested following the G1/S phase of the cell cycle prior to undergoing apoptosis. The aim of the studies reported in this chapter was to establish whether TBP1 function was essential for the 26S proteasome degradation pathway. TBP1 was only recently biochemically identified as being a subunit present in the 19S regulatory complex (Richmond *et al*, 1997). Prior to this it had not been identified in the complex due to its migration pattern in two-dimensional gels; it migrates identically to TBP7 thus it appeared that only 5 ATPases were present in the complex. This may be the reason why very few research groups have examined the role that TBP1 plays in this pathway. The majority of the work pertaining to ATPase function in the regulatory complex has focussed on MSS1, S4, Sug1, and Sug2 proteins. It was hoped that by producing a dominant negative TBP1, the role that this ATPase plays in the complex could be examined. This approach would also allows us to examine the effect of disruption of this pathway more specifically than with the use of the peptide inhibitors.

6.2 Generation of Dominant Negative TBP1 Constructs

Structurally, all of the 6 ATPases associated with proteasome function are highly conserved. The amino-terminal segments of these proteins are more divergent than the carboxy-terminal regions, which has led to the suggestion that the amino-terminal regions may be involved in substrate recognition. Rechsteiner and colleagues (1993) suggested that different ATPases in the 19S regulatory complex may function to recognise distinct substrates for degradation and thus all six

ATPases may not be essential for functional degradation by this complex. To address this question dominant negative TBP1 constructs were generated. It was hoped that this would demonstrate whether TBP1 function was an absolute requirement for this degradation pathway, or whether degradation of a particular set of substrates was disrupted when TBP1 function was lost. The use of trans-dominant negative technology has greatly enhanced the knowledge of biological signalling pathways in the last decade. Previously the preferred method for analysing gene function was to delete the protein of interest and examine the resultant phenotype. In generating a dominant negative protein, a modification is made to the protein of interest to render it non-functional. This approach is of most use when the protein to be examined binds to either another protein or DNA. A mutant can be made in such a manner as to allow binding to its partner or DNA recognition site whilst loosing its functional response. This mutated protein is then introduced into either the *in vitro* or *in vivo* model system. When the mutant protein is expressed at levels in excess of the endogenous protein analysis of the disruption of function of the protein can be examined. One problem with deleting a protein entirely is that it is often not clear whether the complete loss of the protein of interest results in the phenotype observed or if the effects observed are due to disruption of other pathways in which the protein is involved e.g. if the deleted protein, A normally interacts with another protein, B it is often unclear whether protein B will function differently in the absence of protein A. As an example dominant negative IκBα proteins can be produced by mutating the two serine residues that are normally phosphorylated to signal degradation of this inhibitor. These mutated residues result in an IκBα protein that can no longer be phosphorylated thus inhibiting transactivation of the transcription factor NFκB. By producing a modified protein in this manner, it is rendered non-functional but still has the ability to interact with other proteins in this signalling pathway with which it would normally encounter, therefore the phenotype observed will be more relevant to the biological function of this protein.

The approach used to generate a dominant negative TBP1 is shown in Figure 6.1. Structural analysis had previously shown that TBP1 contained a central functional domain, the ATPases module. This analysis also revealed that the amino-

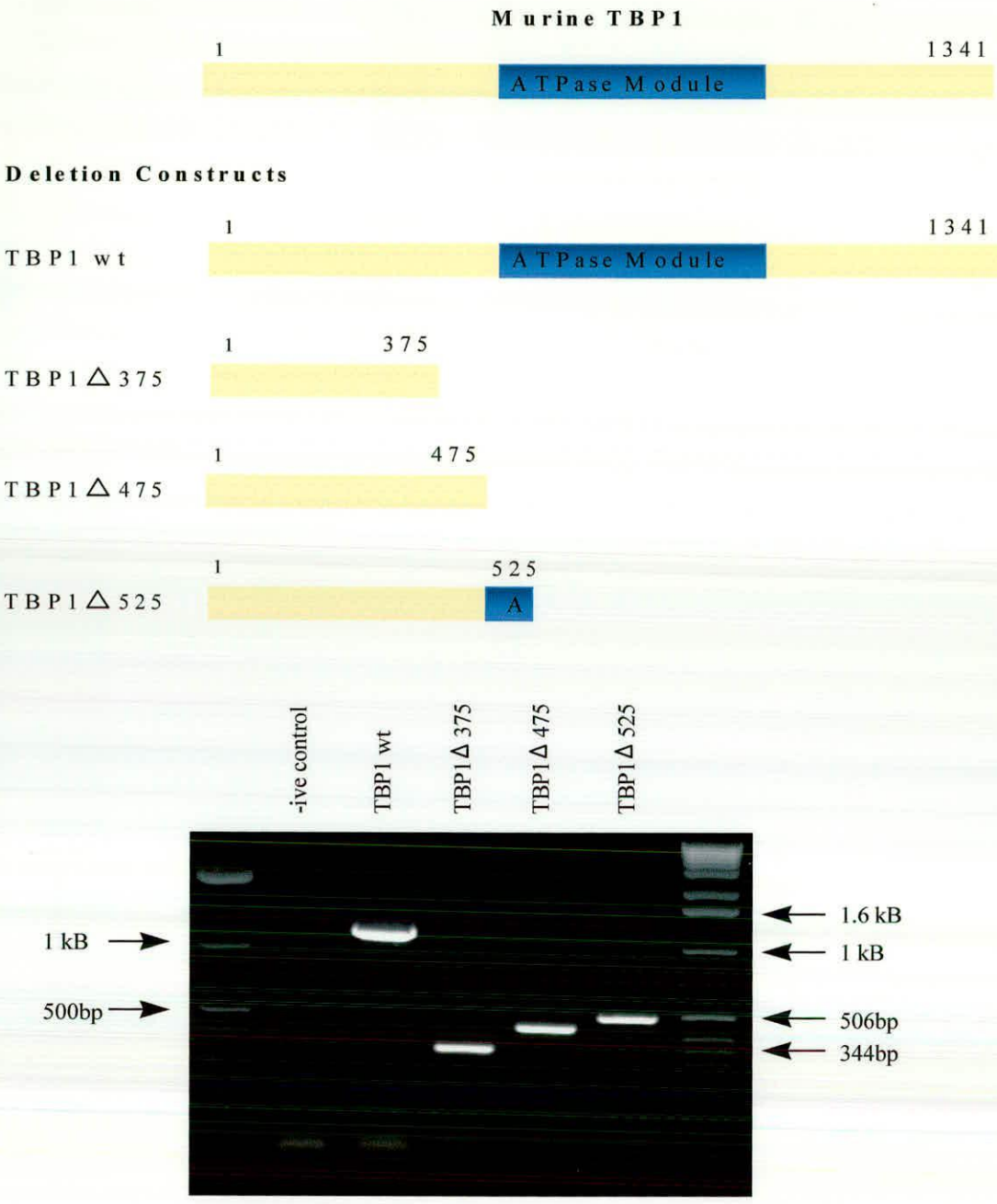
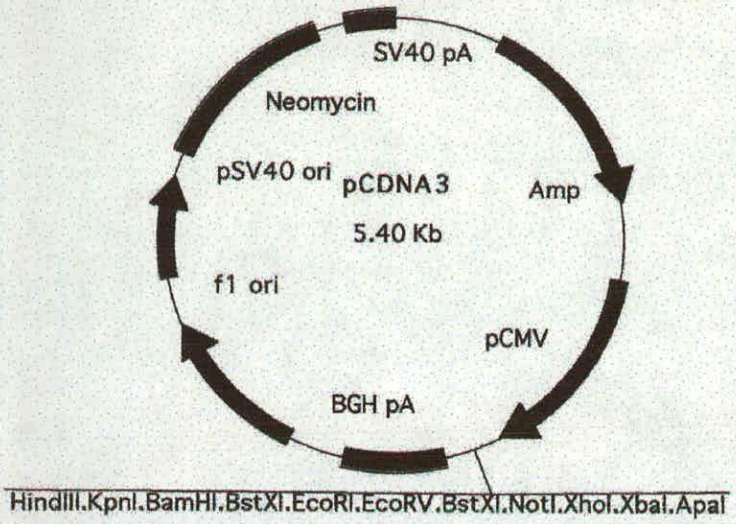


Figure 6.1 TBP1 Deletion Constructs. Truncations in TBP1 were generated by PCR resulting in a number of deletion constructs which either contained 50bp of the functional ATPases module (TBP1 525), terminated at the boundary of the ATPase module (TBP1 475), or terminated 100bp upstream of the ATPase module (TBP1 375). Full length TBP1 (TBP1 wt) was also generated by PCR for ease of cloning. The lower panel represents the TBP1 deletion constructs generated by PCR from day 15 gestation mammary gland cDNA. The negative control reaction was amplified in the absence of template cDNA.

terminal of this family of proteins was most divergent in sequence. This region of the protein contains a leucine zipper-like domain, which had led to the suggestion that this part of the protein is involved in conferring the substrate specificity and may also be involved in protein-protein interactions. Indeed, this region of TBP1 had been shown to be essential for binding to TBP7 (Ohana *et al*, 1993) although an interactions between TBP1 and TBP7 is now disputed. A number of deletion constructs were designed that contained progressively less of the functional domain of this protein. The longest deletion construct generated (TBP1Δ525) contained approximately 30bp of the ATPase module, but this did not include any of the nucleotide binding sites or helicase-like domains which were positioned in the carboxy-terminal region of the ATPase module. The construct TBP1Δ475 terminates 15bp prior to the start of this domain. The shortest construct TBP1Δ375 contained none of the ATPase module, and 100bp of the amino-terminal region was also deleted in this truncated protein. It was postulated that the loss of the ATPase module in these constructs should result in a protein which is no longer functional, but the retention of the amino-terminal should ensure that these mutant proteins can interact with other ATPases to form the 19S cap complex. The deletion constructs were prepared by PCR to allow introduction of restriction sites for ease of cloning (Figure 6.1).

6.3 Expression of Dominant Negative TBP1 Constructs in KIM-2 Mammary Epithelial Cells

The deletion constructs of TBP1 generated by PCR were used for expression studies in KIM-2 mammary epithelial cells. The cloning strategy used is described in Figure 6.2. The PCR products were generated to contain 5' EcoRI and 3' XhoI restriction sites and these cDNAs were ligated to the expression vector pcDNA3. Following transformation, colonies were picked for further analysis. Double stranded sequencing was performed on clones containing inserts to check the correct



EcoRI/XhoI Digested

EcoRI TBP1 Deletion Constructs XhoI

Figure 6.2 Dominant Negative Cloning Strategy. pcDNA3 vector was digested with the restriction enzymes EcoRI and XhoI. TBP1 deletion constructs were generated by PCR to contain 5' EcoRI and 3' XhoI sites. These were subsequently ligated to the vector for transfection into KIM-2 cells.

construct was expressed in the clone chosen for expression in KIM-2 cells. DNA was purified by caesium chloride/ethidium bromide density gradient purification (described in section 2.1.8) prior to transfection into KIM-2 cells.

Many methods have been described for high efficiency gene transfer. These include calcium phosphate precipitation, DEAE-dextran, electroporation, and various lipid based technologies. The preferred method is calcium phosphate precipitation, which is based on the uptake of the foreign DNA into cells in conjunction with a precipitate, mediated by phagocytic vesicles (Graham and van der Eb, 1973). Depending on the cell line used, high transfection efficiencies can be obtained using this method. However, this method has drawbacks, which include a high variability of transfection efficiency between experiments. In general mammary epithelial cells give relatively inefficient transfection efficiencies. This problem was also apparent with KIM-2 cells. Initially the calcium phosphate method was chosen for gene transfer as it had been previously tested in these cells. Proliferating KIM-2 cells were transfected with each of the constructs, and neomycin selection followed 48 hours after the DNA was introduced into the cells. After 14 days selection, no colonies were obtained from these transfections, which suggested that expression of the dominant negative TBP1 constructs was lethal to the KIM-2 cells. This result was not unexpected as the work described in chapter 5 suggested that disruption of the 26S proteasome degradation pathway induced apoptosis.

The lethality of these expression constructs resulted in a different approach being attempted. Ideally an inducible expression system should have been used but, due to time constraints, it was decided to perform a transient tranfection on the KIM-2 cells. This major limitation of this approach is that the maximal transfection efficiency in KIM-2 cells was approximately 0.4%. This would result in too few cells to analyse. To overcome with problem we attempted to increase the transfection efficiency in the KIM-2 cells. A number of transfection reagents were tested, which were mainly lipid based products that were commercially available e.g. Tfx-50 from Promega, Dosper from Boehringer, and Fugene from Boehringer. The results obtained with these products are shown in Figure 6.3. The Fugene reagent

appeared to be optimal for use in KIM-2 cells. It allowed an increase to approximately 0.7% in the transfection efficiency, which on average was twice as efficient as the calcium phosphate method. The major advantage of the Fugene product was that the transfection efficiency initially appeared to be reproducible, which is often not the case for the other methods used. Although the efficiency of this procedure had been increased, it did not result in a high enough number of transfected cells to analyse a phenotype e.g. if expression of a dominant negative TBP1 induced apoptosis, then only 0.7% of the cells would display this effect, and this would be difficult to discriminate from background death as a result of the transfection procedure. With this in mind, a system was chosen for use that would allow purification of transiently transfected cells. The Capture-Tec kit from Invitrogen allows the rapid selection of transiently transfected cells from total populations of both transfected and untransfected cells. This is achieved by virtue of a specially designed vector (pHook), which expresses a single chain antibody (sFv) against a specific hapten on the surface of transfected cells. Transfected cells can then be isolated from the total cell population by binding to hapten coated magnetic beads. Co-transfection of this vector with the construct of interest allows isolation of cells expressing the gene of interest within as little as 2 hours post-transfection. This system was designed to allow for study of the effects of dominant negative mutations on growth regulating proteins or toxic gene products, and should allow the analysis of transient transfections in mammary epithelial cells.

Further optimisation of the Fugene transfection method revealed inefficiencies within this system. In general the transfection efficiency appeared to be extremely sensitive to the template DNA quality. Only DNA which had been twice purified by CsCl density gradient centrifugation would transfect with the Fugene reagent. Batch differences in the template DNA also reduced the efficiency of this method e.g. one sample of a test plasmid, which was a LacZ reporter (pCH110), would give a successful transfection, whereas a different batch of the same plasmid produced in an identical manner, but at a different time would give no transfected cells at all. This problem was partially overcome by dialysing the purified plasmid for 30 minutes against ddH_2O on a 0.05μM millipore filter prior to

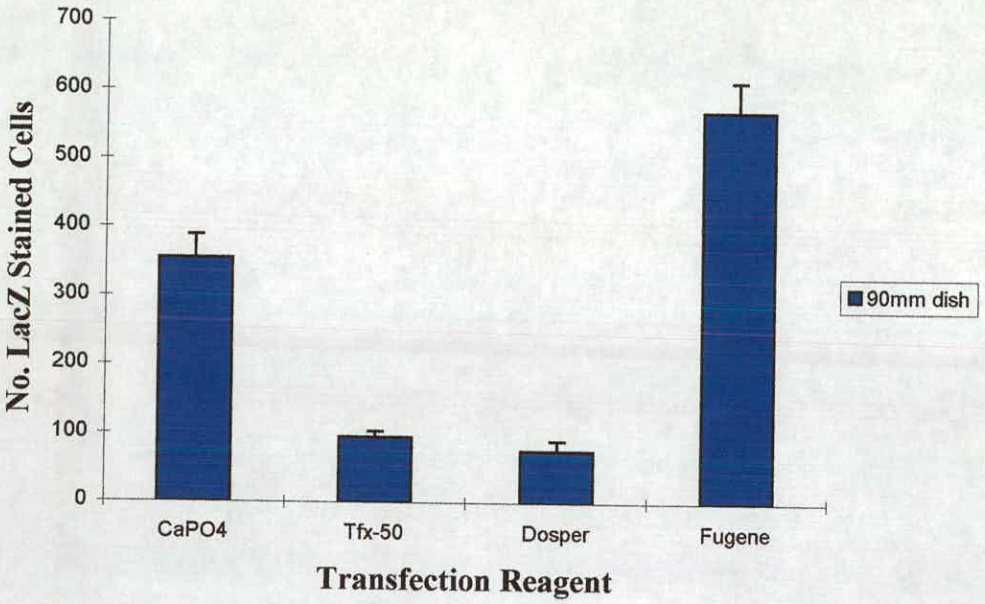
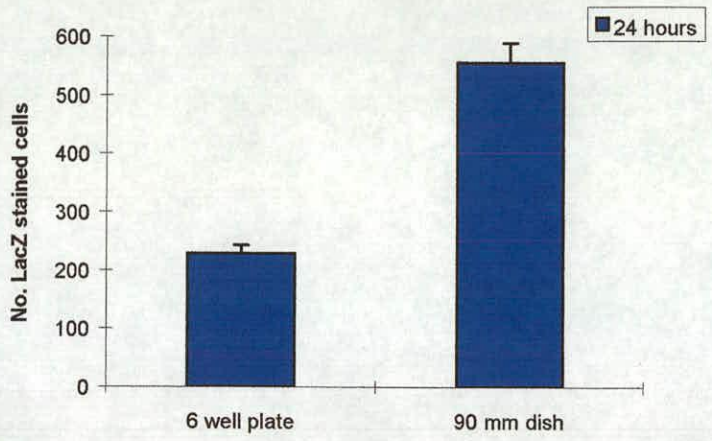


Figure 6.3 Comparison of Transfection Efficiencies in KIM-2 Cells using a Variety of Transfection Methods. KIM-2 cells were passaged 24 hours prior to transfection. Cells were transfected according to the manufacturer's instructions for each reagent, using a lacZ reporter plasmid, pCH110. Cells were transfected for a full 24 hours prior to fixation and staining. The number of blue cells per dish were counted and are expressed as the mean of three experiments ± sem.

the transfection. Initial transfections using Fugene were performed in six well plates over a 24 hour period. Ideally to express the TBP1 deletion constructs a large number of cells would have to express the construct to allow purification using the pHook system. Figure 6.4a shows results of experiments where the number of cells transfected were increased and the effect this had on the transfection efficiency monitored. Although approximately twice as many cells were transfected in a 90mm dish as compared to the 6 well plate, the large dish contained six fold more cells and the subsequent transfection efficiency rate was lower. These results were obtained when using 2μg DNA transfected in 100μl diluted Fugene reagent in both samples. When the concentration of DNA was either increase or decreased (Figure 4.6b(i)) or the amount of Fugene reagent was increased, a decrease in the transfection efficiency was observed. This was also apparent when the number of cells plated was reduced (Figure 4.6b(ii)). From these experiments it appeared that the Fugene reagent worked optimally when 2μg DNA was added to 3μl Fugene diluted to 100μl in medium. This created a potential problem with the use of the pHook system. It is recommended to transfect ten fold less of the pHook vector than test plasmid to increase the probability of purified cells expressing pHook in addition to the test plasmid. This would results in 2μg test plasmid transfected with 0.2μg of the pHook vector. This made it seem highly likely that very few cells would actually express the pHook vector in addition to the deletion construct due to the small amount of DNA used.

Further optimisation of this system was required as a 24 hour transfection period was too long; the mutant TBP1 constructs were predicted to be lethal when expressed in the cells, and would very likely be expressed within this 24 hour period. The duration of Fugene transfection, using the LacZ reporter pCH110 was decreased from 24 hours to either 2 hours or 5 hours and the cells assayed for lacZ activity (Figure 6.4c). These results determined that the transfection efficiency was very low in cells following these short transfections. On average, fifty cells would stain positive for LacZ expression after 5 hours; this was too few cells for use with the pHook system. Logistically this resulted in it being impossible to perform the transient assay and purify the cells using the pHook vector as had been hoped.

(a)



(b)

(i)

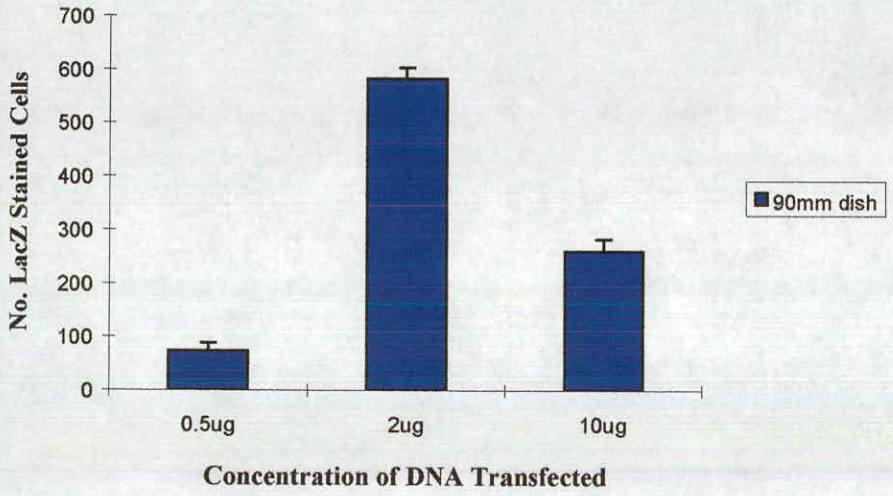


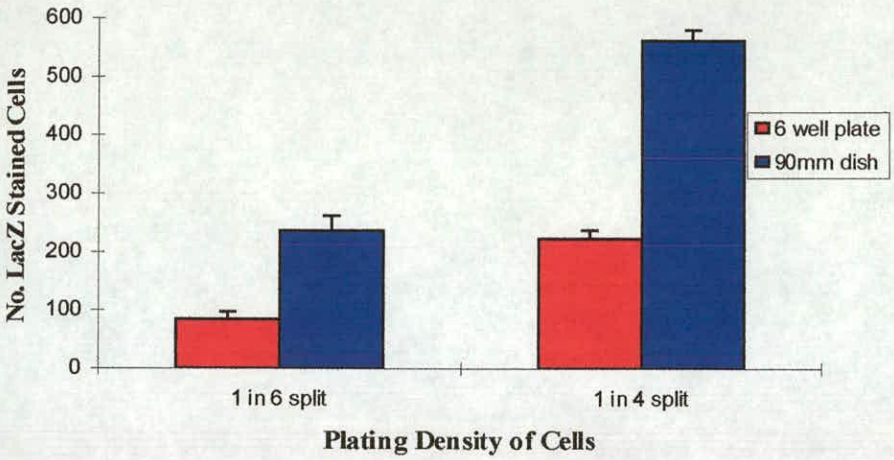
Figure 6.4 Optimisation of Fugene Transfection Reagent.

(a) Effect of Cell Number on Transfection Efficiency. KIM-2 Cells were passaged 24 hours prior to transfection. 2μg of test plasmid (pCH110) was mixed with 100μl diluted Fugene reagent and added to the cells for 24 hours. Cells were subsequently fixed and stained and the number of LacZ positive cells counted. The results shown are the mean of three experiments ± sem.

(b) (i) Effect of DNA Concentration on Transfection Efficiency. KIM-2 cells were passaged 24 hours prior to transfection using three different concentrations of test plasmid (pCH110). Cells were transfected for 24 hours prior to fixing and staining. The results represent the number of cells stained positive for LacZ expression, and are expressed as the mean of three experiments ± sem.

(b)

(ii)



(c)

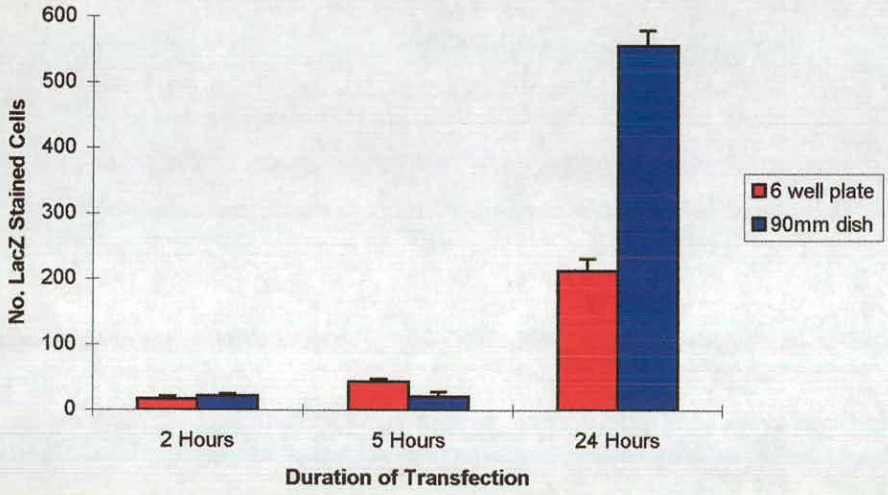


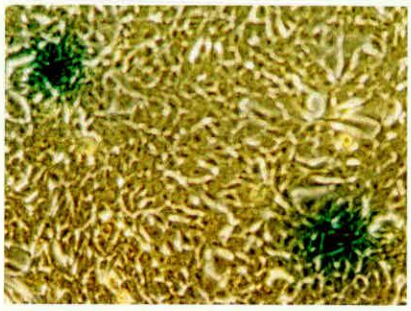
Figure 6.4 (b) (ii) Effect of Plating Density on Transfection Efficiency. KIM-2 cells were passaged into either 6 well plates or 90mm dishes at either a 1in 4 or 1 in 6 split (1 in 4 corresponds to $0.66x10^6$, or $0.66x10^7$ cells in a 6 well plate, or a 90mm dish, and $1x10^6$, or $1x10^7$ cells at a 1 in 6 split in a 6 well plate or 90mm dish respectively). Cells were transfected with 2μg pCH110 over 24 hours, prior to fixation and staining of the cells. The results represent the number of cells stained positive for LacZ expression, and are shown as the mean of three experiments ± sem. **(c) Effect of Duration of Transfection on Transfection Efficiency.** KIM-2 cells were passaged as normal 24 hours prior to transfection. Cells were transfected with 2μg DNA added to 100μl diluted Fugene reagent over a 24 hour period. Cells were subsequently fixed and stained for LacZ expression. The results are expressed as the mean of three experiments ± sem.

Although all of these expression experiments had failed to produce cells expressing mutant TBP1 proteins it is not difficult to reconcile this with the lethality of TBP1. To attempt to address the question of lethality of mutant TBP1 in KIM-2 cells a transfection was performed using the deletion constructs in conjunction with the lacZ reporter construct pCH110. KIM-2 cells were transfected using Fugene with 2μg TBP1 deletion construct in addition with 0.5μg pCH110. It was assumed that if expression of these TBP1 proteins were lethal to the cells then no cells expressing lacZ would be detected. Results of this experiment are shown in Figure 6.5. Expression of all three deletion constructs, in addition to wild type TBP1 was lethal to KIM-2 cells. In contrast to this, in cells transfected with a control vector (pBluescript) lacZ stained cells were observed. These results are highly suggestive of these constructs being lethal when expressed *in vitro*. Overexpression of TBP1 alone appeared to be lethal to the cells and to answer this question more empirically we reversed the concentration of lacZ reporter and TBP1 construct. This results in a small number of lacZ stained cells being detected suggesting that expression of TBP1 at high concentrations in KIM-2 cells is lethal. Whether the effect of overexpression of TBP1 is due to disruption of protein degradation cannot be addressed within this system.

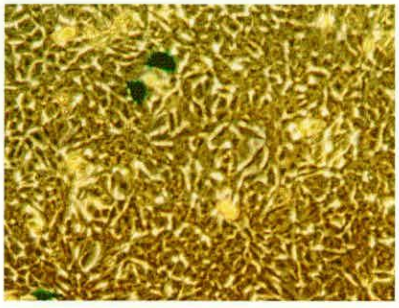
6.5 Discussion

The results presented in this chapter suggest that TBP1 is an essential component within the cell. These results are an indication that at least the ATPase TBP1 is required for proteasome function, which itself suggests that the function of all six ATPases may be a pre-requisite for proteasome function. If this was indeed the case then it would suggest that these proteins co-operate to unfold and degrade proteins, and it would seem highly unlikely that they act to process different substrates as has been previously suggested (Dawson *et al.*, 1995). If these ATPases each act to degrade a different set of target proteins then it would be unlikely that an

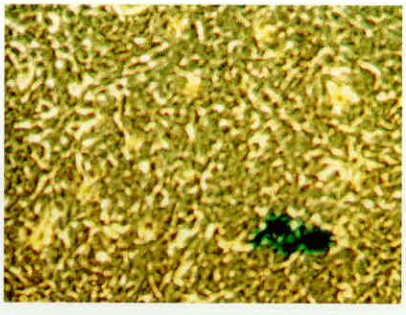
Figure 6.5 Expression of TBP1 Deletion Constructs in KIM-2 Cells. Cells were passaged 24 hours prior to transfection using the Fugene reagent. Cells were transfected with either a lacZ reporter construct alone (pCH1110), with pCH110 in addition to an unrelated control vector (pBluescript), or with pCH110 in addition to either wild type (wt) or deletions constructs of TBP1. Cells were transfected for a full 24 hours prior to fixation and staining. The figures in brackets express the average number of lacZ stained cells ± sem per dish (approximately 1-1.5 x 10^7 total cells per dish). The results shown are expressed as the mean of three experiments.



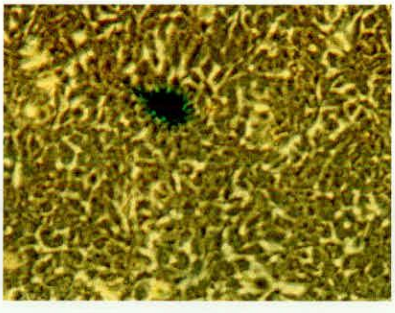
0.5µg pCH110 (160 + 8.326)



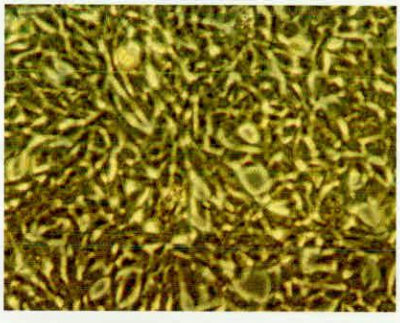
2µg pCH110 (298 + 9.905)



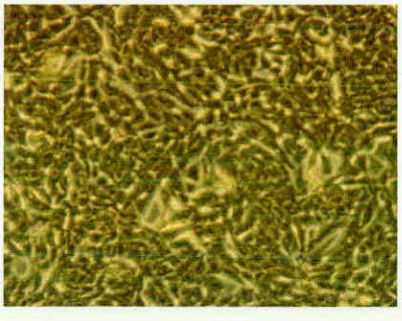
2µg pBluescript + 0.5µg pCH110 (127 + 6.692)



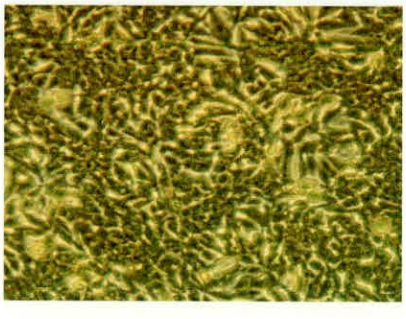
2µg pCH110 + 0.5µg TBP1 wt (84 + 3.561)



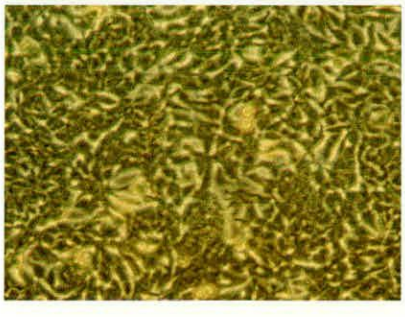
2µg TBP1 wt + 0.5µg pCH110 (0)



2µg TBP1Δ375 + 0.5µg pCH110 (0)



2µg TBP1Δ475 + 0.5µg pCH110 (0)



2µg TBP1Δ525 + 0.5µg pCH110 (0)

identical phenotype would be observed when two different subunits are deleted. Disruption of S4 in *S. pombe* has shown that this ATPase is essential for survival (Gordon *et al.*, 1993). The results presented in this chapter suggest but do not confirm that TBP1 is essential for proteasome function, and that loss of this protein cannot be compensated for by other ATPases. It was hypothesised that a degree of redundancy may exist in this system due to the high level of conservation between these proteins. Since disruption of TBP1 function is highly likely to be lethal (as for S4 in yeast) this suggests that other proteins, particularly ATPases, could not compensate for this loss of function.

The studies in KIM-2 cells showed that loss of the functional ATPase module resulted in the inability to obtain clones that expressed the mutated constructs suggesting that this may be lethal to the cell. The studies performed in MEC were preliminary, and problems were encountered due to inefficient transfections in KIM-2 cells. HC11 MEC have been transfected with much higher efficiency than KIM-2 cells, but for this study HC11 cells could not be used due to their loss of functional p53. Results presented in chapter 5 demonstrated that MG132, although inhibiting degradation of ubiquitinated proteins, had no effect on the rate of apoptosis in HC11 cells, excluding their use in the transfection studies. Several fundamental problems were encountered when transfecting KIM-2 cells. Mammary epithelial cells are plastic in nature e.g. KIM-2 cells are thought to contain mammary gland progenitor cells. These cells have the ability to form either myoepithelial or luminal epithelial cells. KIM-2 cells have also been observed to spontaneously transform to cells that are more spindle-like in appearance. In general epithelial cells are highly dependent on cell to cell contacts. When clones are isolated cells lose this contact, which results in a number of the cells losing an epithelial phenotype. With time these cells expand and occupy much of the clone and therefore these clones have to be excluded from analysis. For these reasons large numbers of clones have to be selected for analysis to obtain a number of clones which appeared and behaved identically to the starting population. As analysis on cloned KIM-2 cell lines proceeded it became apparent that some cultures were loosing their ability to respond to lactogenic hormones. As with the changes in morphology observed, the degree of response to

lactogenic hormone expression changed. Some clones expressed β-casein to a similar level as in the uncloned cells, whereas some lines expressed less milk protein, and other lines did not express any β-casein. The problems with transfection efficiencies will be difficult to resolve in KIM-2 cells. The cells used in this thesis are currently the only MEC which can be induce to express the late differentiation marker WAP when grown in the absence of extracellular matrix. When these cells differentiate they form mammospheres or domes, which are comprised of an inner layer of epithelial cells with some myoepithlial cells present on the outer layer. This structure is very similar to the alveoli *in vivo*. The similarity in structure to the mammary gland is hypothesised to contribute to the differentiation capacity of these cells, which make KIM-2 cells the most relevant *in vitro* model of mammary gland differentiation.

It could be assumed that transfection of the TBP1 dominant negatives into the KIM-2 cells resulted in a lethal phenotype from the inability to express these constructs in transfected cells. Since no stable cell lines could be generated, this reduced the ability to fully analyse the effect of disruption of this pathway and too few transiently transfected cells were available. In retrospect it may have been more prudent to choose an inducible system to analyse the TBP1 dominant negative constructs in KIM-2 cells. At the start of this study the tetracycline-regulated system would have been the ideal choice. The system is based on a tetracycline-controlled transactivator (tTA), which combines the tetracycline repressor (tetR) DNA binding domain with the transcriptional activation domain of VP16 from the herpes simplex virus. The gene of interest is cloned downstream of a minimal promoter fused to seven repeats of the tet operon (tetO). When tTA binds to the tetO, transcription of the target gene is activated. In the presence of the antibiotic tetracycline, binding to tTA results in a conformational change that prevents binding to the tetO. The removal of tetracycline results in gene activation (Schockett *et al.*, 1995). A reverse system is also available where mutations in the tetR result in binding to the tetO only in the presence of tetracycline. This system thereby allows the induction of gene expression by either the addition or the removal of tetracycline from the medium depending on the vectors used. The major advantage of this system is that

tetracycline is not toxic at the concentrations required to induce/repress expression, the induction of gene expression is extremely rapid and expression of the regulated gene is very low in the uninduced state (Schmid, 1995). This system is based on producing a tTA stably expressing cell line, which can then be transfected with the gene of interest. The intermediate tTA expressing cell lines require to be tested for inducibility using a tetO reporter plasmid before further use. Within KIM-2 cells the tetracycline-regulated system would again be troubled with the transfection problems discussed above, and would require a lengthy period of optimisation for MEC. At present we have a KIM-2 cell line expressing the Tet repressor. Results from our laboratory and others have shown substantial problems with this system. The KIM-2 cells expressing the Tet repressor do not show regulated expression e.g. these cells are leaky in expression resulting in the continual expression of the construct as opposed to the ability to switch on expression after stable clones have been generated. This is clearly a potential problem when disruption of a gene is expected to be lethal, as is the case for TBP1. In hindsight it was prudent not to have spent the time optimising expression using the tetracycline system as it would have proven impossible to generate cell lines stably expressing my TBP1 deletion constructs. Additional inducible expression systems are available, the ecdysone system being the most preferred of these. This system functions in a similar manner to the tetracycline system allowing tight regulation of expression will very low basal levels of expression. At present there are very few documented reports of the use of this system therefore it remains to be seen whether it will be more tightly regulated than the Tetracycline system. Even if the ecdysone system proves to be more regulatable than other inducible systems, the cloning problems inherent to mammary epithelial cells will still be encountered. At present the approach now favoured within our laboratory is the use of new generation retroviral vectors. When packaged, these vectors can result in high infection levels in cells and it is hoped that this will overcome the previous cloning problems encountered.

The results presented in this chapter have demonstrated that TBP1 function may be essential for cell viability in KIM-2 MEC. This mirrors the results obtained using a chemical inhibitor of this degradation pathway. These results taken together

strongly suggest that degradation of ubiquitinated proteins is a pre-requisite for cell survival and that disruption of one component, TBP1, within the regulatory component of this complex is all that is required to disrupt this function.

Chapter 7:

Discussion

7.1 Summary

The results presented in this thesis detail an essential function for the ubiquitin-dependent protein degradation pathway in mammary epithelial cells. As part of these studies I optimised differential display for use in the mammary gland. This work describes the first isolation of the murine homologue of TBP1 and describes an absolute function for the 26S proteasome in the survival of a differentiated tissue. Studies in mammary epithelial cells suggested that disruption of this degradation pathway using either a chemical inhibitor of the 26S proteasome or a dominant negative construct of TBP1 was lethal to these cells. An apoptotic response was induced that was shown to be cell cycle stage dependent in addition to being dependent on the presence of the tumour suppressor protein p53. Disruption of TBP1 function was assumed to generate an identical response to that induced by MG132 due to the inability to select clones expressing these mutant constructs. These results suggest that TBP1 is an essential component of the ubiquitin-dependent protein degradation pathway. The following sections discuss in more detail the regulation of protein degradation in the mammary gland and how this pathway may act to control cell cycle progression. I will also discuss future experiments required to answer some of the outstanding questions.

7.2 Regulation of Proteolysis in the Mammary Gland

The expression of the 26S proteasome increases from day 10 gestation onwards in the mammary gland. The reason why such an increase in the proteolytic machinery is required in late gestation is currently unknown, however this is the particular stage in gland development when the differentiation process is initiated, indeed it is when the gland first expresses the milk protein β-casein. The relevance

between the onset of milk protein expression and the increase in proteasome expression levels may be coincidental but the possibility exists that the proteasome is required to degrade milk protein prior to secretion. By late gestation β-casein mRNA accounts for around 20% of the total mRNA present in the cell. Whilst massive amounts of milk protein are being produced by the gland prior to parturition none of this protein is secreted. This function is thought to prepare the gland for lactation but the precise mechanism that prevents secretion of milk is unclear. *In vitro* studies on purified 20S proteasomes have shown that β-casein can be degraded by this complex (Pereira *et al.*, 1992). Whether casein can be degraded *in vivo* by the 26S proteasome is not known. It is equally possible that another proteolytic system acts to remove milk protein from the cell prior to lactation. Very little information is published with regard to proteasome expression in developing tissues, but the little that is known suggests that there are at least changes in localisation of the 20S proteasome when a developmental process is initiated. In general, expression is found to be nuclear in rapidly proliferating cells whereas cytoplasmic staining is more predominant in quiescent cells. Mechanisms such as protein degradation are thought to allow a cell to adapt to changes in signalling which are encountered during development. The increase in proteasome expression in the mammary gland may be a consequence of the progression of the cell towards a differentiated phenotype. An increase in the metabolism of the cell is also expected to occur when the cell differentiates. It is possible that an increase in protein degradation may be a consequence of the cell gaining a more specialised function such as the mammary cell does during pregnancy. This is in contrast to the decrease in the 20S proteasome during lactation and involution. The ability to alter the cellular environment by the removal of proteins through upregulation of the degradation machinery should promote a cell efficiently along the pathway towards gaining a specialised function. Whether this can account for the increase in proteasome function in the mammary gland during pregnancy is purely speculative. This theory may also fit with the observation of a decrease in the 20S proteasome early in the involution process. It would appear that the levels of this degradation machinery are of less importance as the structure of the gland remodels. The major

proteases involved in restructuring the mammary gland at this stage are the matrix metalloproteinases (section 1.3.5.1) thus there may be a lesser requirement for any additional proteolytic machinery during this process. In order to address this question studies on the localisation of the proteasome during pregnancy and lactation are required. In hindsight it would have been extremely valuable to raise an antibody against TBP1. When good antibodies become available against the proteasome components, the function this complex plays during development may become clearer. The study described in this thesis is one of the first studies of proteasome expression in developing tissues. At present most of the research on proteasome function is based on biochemical purification of its components or yeast mutant analysis. Ideally, the question of TBP1 function in the developing mammary gland could be answered more empirically if a mouse model of TBP1 disruption was generated. The use of tissue specific, temporal specific technologies such as the lox/Cre recombination system would allow the examination of proteasome function in the mammary gland during its cycles of proliferation, differentiation and involution. What appears clear from the studies using mammary epithelial cells is that disruption of the proteasome in an actively proliferating cell results in a more severe apoptosis than disruption in a differentiated cell. The generation of a mouse carrying a dominant negative TBP1 construct would allow the expression levels of the major proteins involved in gland development to be examined when the proteasome is disrupted.

The 26S proteasome has been implicated in processing and degrading a number of proteins that play a controlling role in mammary development. One of these is the Jak/Stat pathway, which signals through a range of cytokine receptors to control mammary development during pregnancy, lactation, and involution. This signalling pathway has recently been observed to be regulated by the proteasome. Kim and Maniatis (1996) have shown that proteasome inhibition of HeLa cells stimulated by IFN-γ results in the ubiquitination and stabilisation of Stat1. In general the phosphorylation and nuclear binding of Stat1 is maximal 15 to 30 minutes following stimulation. The levels of Stat1 are then seen to rapidly decrease to

undetectable levels within 1 to 2 hours. The regulation of this response is of prime importance to the cell as it must be able to respond to stimulation from numerous cytokines simultaneously. Without this regulation the cell would rapidly accumulate toxic amounts of gene products and be unable to coordinate the cytokine responses. These studies demonstrated that phosphorylation of Stat1 was a prerequisite for its ubiquitination. In contrast to this, stimulation of Stat3 and Stat5 levels by interleukin-6 (IL-6) were unaffected in a hepatoma cell line treated with proteasome inhibitor (Rayanade *et al.*, 1997). This study suggested that p53 played a role in protecting these transcription factors from proteasome degradation. It was hypothesised that in the presence of p53, a gene product was produced that masked Stat3 and Stat5, but not Stat1 from being degraded by the 26S proteasome. More recently Callus and Mathey-Prevot (1998) have shown that IL-3 stimulation of the Jak/Stat pathway results in prolonged activation in the presence of proteasome inhibitors. This group observed sustained phosphorylation of Stat5 when the 26S proteasome was disrupted. They found no evidence to suggest that Stat5 was inactivated by this pathway and its prolonged activity was due to persistent phosphorylation of Jak2. The mechanism by which the proteasome normally deregulates Jak activity is unknown but it has been suggested that its action may be indirect. It may function to process a phosphatase that acts to dephosphorylate the Jak proteins. In the mammary gland Stat5 is active during late pregnancy and lactation and stimulates milk protein gene production in response to prolactin. Stat3 and Stat1 are found elevated during involution in the gland suggesting that they act either positively or negatively to control the apoptotic response of the gland. The increase in the 26S proteasome in the mammary gland cannot be accounted for simply by the increased requirement for the control of the Jak/Stat pathway. The increase in expression of these genes do not overlap and it seems unlikely that such a substantial amount of Jak/Stat signalling occurs in the gland that a ten-fold increase in the proteasome is required to counteract this. If a mouse model of proteasome disruption were available then the precise regulation of these signalling molecules could be examined. Another approach to gain knowledge on the function of proteasome regulation of Jak/Stat proteins is to generate non-degradable forms of

these proteins and examine the consequences of prolonged action. Absence of Stat5a in the mammary gland results in an inability to lactate therefore it seems reasonable to assume that prolonged activation of this protein would result in an elongated lactation period. It would be interesting to examine whether these mice would be able to undergo a successful involution, which would give details on whether Stat5 could act as a survival factor for the mammary gland. This is of interest to proteasome function as it is unclear whether the apoptotic response observed in response to proteasome inhibition is simply due to deregulation of the cell cycle or prolonged activation of pro-apoptotic factors.

The transcription factor NFκB is another protein that is developmentally regulated during mammary gland development. It is highly active, as measured by DNA binding activity during the latter half of gestation, is absent during lactation and its activity is high again during involution (Dr R. Clarkson, pers. comm.). The exact function this protein exerts on mammary gland development is not clear. The proteasome controls NFκB in two ways: it proteolytically cleaves p105 to produce the active p50 component and it also ubiquitinates and degrades the inhibitor IκBα. NFκB has been suggested to function as a factor which can protect a cell against apoptosis. In T cells stimulated with TNFα, NFκB was observed to prevent an apoptotic response to a subsequent stimulus (Liu *et al.*, 1996). Whether it plays this type of role in the mammary gland is unclear. Its expression profile may suggest that it acts in this manner as very little apoptosis can be observed in the gland in late gestation. Involution is characterised by a two stage response. The initial stage is characterised by apoptosis of the epithelial cells, and this stage is reversible. It may be that NFκB is highly expressed at this stage to ensure that an anti-apoptotic response can be initiated if required. It would seem logical to speculate that if the proteasome is disrupted then no active NFκB would be produced and this would result in the loss of a protective factor to the cell. This in itself may stimulate an apoptotic response if no other protective factors were present within the cell. *In vivo* it would seem highly unlikely that the increase in the proteasome is as a response to an increase in active NFκB. The expression profiles of these proteins do not fully

overlap e.g. the proteasome, like NFκB is expressed highly in the latter half of gestation, but it is also expressed highly during lactation when NFκB activity is low. When additional information is available on the function of NFkB in the mammary gland then it may be easier to judge whether disruption of this function would be lethal.

Cyclin D1 is known as a target of this protein degradation pathway. Mice lacking cyclin D1 show no proliferation in response to steroid hormones during pregnancy (Sicinski *et al.*, 1995). Within this thesis we have shown that the 26S proteasome is essential to the function of a proliferating epithelial cell. Whether the apoptosis observed following inhibition of this pathway is as a direct consequence of perturbing cyclin D1 expression is unclear. One way to address this question would involve producing a non-degradable form of cyclin D1. Using this approach the direct effect of overexpression of this protein alone can be examined.

7.3 The 26S Proteasome as a Mechanism for the Regulation of Apoptosis

A major portion of the work described in this thesis detailed the apoptotic response induced by proteasome inhibition in mammary epithelial cells. Treatment with the proteasome inhibitor MG132 induced an apoptotic response that was dependent on the presence of p53 and occurred following the G1/S phase of the cell cycle. Examination of the cdk inhibitor p27 revealed elevated expression suggesting that this protein, which controls entry into S phase, was deregulated. This suggested that cells were traversing the G1/S phase prior to undergoing apoptosis. It was hypothesised that inhibition of the ubiquitin-dependent protein degradation pathway would result in the deregulation of a number of proteins that control progression of the cell cycle and this would be a trigger for the apoptotic response observed. Examination of each of the major control proteins for the cell cycle should reveal the

exact nature of the apoptosis induced.

Treatment of KIM-2 cells with cell cycle blocking agents demonstrated that cells were able to traverse the G2/M boundary when this pathway was disrupted, although at a much slow rate than untreated cells, but were unable to progress forward from G1/S phase. The data regarding proteasome inhibition on cell cycle status is conflicting at present. It appears that dependent on the cell type used a G1/S phase and/or a G2/M block can be induced. In this study treatment of ES cells with MG132 was observed to induce a G2/M block followed by apoptosis in p53wt ES cells, which contrasted to that observed in the KIM-2 cells. Identical results to those described in this thesis for KIM-2 cells have been obtained in T cells. It was observed that proteasome activity was essential for cells to progress from the G1/S boundary to the G2/M phase, but not essential from the progress from G2/M phase to the next G1 phase (Wang *et al.*, 1998). In contrast to this, examination of the cell cycle kinetics in African trypanosomes demonstrated that procyclic forms of the parasite required the proteasome for progression from G2, whereas bloodstream forms could not progress from either G1/S phase or from G2/M when the proteasome pathway was blocked (Mutomba *et al.*, 1997). These studies would suggest that cell cycle arrest by proteasome inhibition is dependent on the cell type and the cellular processes occurring at the time of inhibition.

The induction of apoptosis following G1/S phase in KIM-2 cells led us to examine this particular stage of the cell cycle. Examination of the cdk inhibitor p27 demonstrated that elevated levels of this protein were present. p27 is a protein that controls the progress of cells from the G1 phase of the cell cycle into S phase. Elevated levels of any of the cdk inhibitors have been shown to induce apoptosis *in vitro*. It is possible that proteasome inhibition induces apoptosis as a direct consequence of perturbing the expression of p27 but it would seem much more probable that the apoptosis observed was as a consequence of the deregulated expression of a number of cell cycle control proteins. The proto-oncogene c-*myc* is another target of the ubiquitin-dependent degradation system. Expression of c-myc

can induce either a proliferative or apoptotic response, which is dependent on the cellular environment. Generally levels of c-myc are elevated at the onset of G1 and this triggers the progression of the cell through this stage of the cell cycle. As the cell reaches late G1 c-myc levels fall and the levels of p27 rise. These events are thought to help trigger the entry of the cell into S phase. Elevated levels of c-myc prevent S phase entry and have been observed to induce apoptosis (Evan *et al.*, 1992). In progression of the cell cycle the levels of c-myc and p27 appear to be opposing. With this is mind we examined the levels of c-myc protein in KIM-2 cells to determine if both c-myc and p27 were elevated. The results generated were inconclusive as the antibody used was non-specific. This needs to be repeated to determine if there are conflicting levels of these two proteins.

More recently another cdk inhibitor p21 has been shown to be regulated by the 26S proteasome (Wang *et al.*, 1998). p21 increases in expression in response to p53 and triggers arrest of the cell cycle at G1 (Gartel *et al.*, 1996). Its expression is normally tightly regulated and it can respond in both p53-dependent and independent manners. The apoptosis observed as a direct consequence of proteasome inhibition is p53 dependent suggesting that p21 may be activated in response to this signal. Proteasome inhibition has been previously shown to suppress the upregulation of p21 during G1 suggesting that the proteasome might facilitate cell proliferation by augmenting expression of p21 during the G1 phase of the cell cycle. Modulation of p21 expression would hamper the entry of the cell into S phase and any progression from this stage, which is similar to the results observed with proteasome inhibition.

The E2F family of transcription factors also contributes to cell cycle regulation and are substrates of the proteasome. E2F proteins interact with the pRb pocket proteins, and this interaction functions to inhibit the DNA binding activity of E2F in addition to masking it from degradation by the proteasome. Phosphorylation of pRb releases E2F allowing it to promote entry into S phase of the cell cycle. Various E2F/pocket protein complexes exist and these are thought to perform different tasks as they are present at different stages of the cell cycle. E2F and the

pocket protein p130 are found predominantly in both quiescent and terminally differentiated cells, E2F/p107 complexes are present mostly in S phase, and E2F/pRb in early to mid G1 (reviewed in Bernards, 1997). E2F-1 has been shown to be a specific substrate for the 26S proteasome. Although the majority of E2F proteins can act to induce entry into S phase, E2F-1 is the only family member that can induce apoptosis. Disruption of protein degradation by the ubiquitin-dependent proteasome pathway should result in overexpression of E2F, which can induce S phase entry followed by apoptosis (Shan and Lee, 1994). This is similar to the response observed in KIM-2 cells treated with MG132 but it may not be the direct cause of the apoptotic response. KIM-2 cells express SV40 Tag which complexes pRb. How this affects pRb binding to E2F-1 is unclear but the results observed in KIM-2 cells at 33°C and ES cells and would suggest that p53 is a major component of the response to proteasome inhibition. In addition to controlling S phase entry E2F has a number of target genes which contribute to cell cycle regulation. A high level of free E2F in late G1 initially suggested that its prime role was to activate genes during that period. These genes included cyclin A, cyclin E, the cdk inhibitor p16, p107, pRb, E2F-1 and the nuclear proto-oncogenes *c-myc*, N-*myc*, and B-*myb* (Beijersbergen and Bernards, 1996). Analysis of the promoters of these genes revealed that E2F elements in general act as negative elements during G1 as opposed to positive elements during S phase. Disruption of the 26S proteasome degradation pathway should result in elevated levels of E2F. This could equally be a consequence of pRb binding to SV40 Tag. This may further cause deregulation of its target genes which themselves are cell cycle regulated. Indeed some of the E2F targets are also degraded by the proteasome suggesting that by altering the expression of one protein a number of cell cycle proteins can be disrupted.

p53 levels were observed not to increase as a result of proteasome inhibition in this study. This observation conflicts what is currently known with regard to p53 degradation. It is well documented that p53 is degraded by the 26S proteasome *in vivo*. Ubiquitinated species of p53 have been observed in cells *in vitro* confirming this theory (Maki *et al.*, 1996). p53 association with the oncoprotein mdm2 has been

observed to affect the susceptibility of this protein to ubiquitin mediated degradation. I have shown that although the presence of p53 is necessary for the initiation of an apoptotic response, elevated levels of p53 do not initiate this reaction. It has been observed in many situations that deregulation of p53 can induce an apoptotic response. If this protein was indeed degraded by the proteasome then it would have been the obvious conclusion to have reached e.g inhibition of the 26S proteasome should result in elevated levels of p53 which alone could be the trigger to induce apoptosis. The exact mechanism which prevents a stabilisation of p53 in the presence of MG132 in KIM-2 cells is unclear but may be due to the presence of SV40 Tag in these cells. Initially it was assumed that Tag should not affect the degradation of p53 as it had been observed that HPV infected cells can degrade p53 in the presence of SV40 Tag (Scheffner *et al.*, 1990). It is possible that another proteolytic pathway may regulate p53 stability. Recently it has been suggested that calpains may also play a role in the degradation of p53. Calpains are calcium-dependent intracellular proteases which are divided into two major groups: the ubiquitous calpains, termed milli- and microcalpains according to the concentration of calcium required for their activity, and tissue specific calpains (Croall and DeMartino, 1991). Ubiquitous calpains, which are predominantly cytoplasmic in nature, recognise proteins by structural determinants and cleave their substrates only partially. Pariat and colleagues (1997) demonstrated that p53 was susceptible to calpain proteolysis and that this degradation was dependent on p53 protein conformation. Wild-type p53 protein acts to either promote or suppress cell proliferation and these opposing functions correlate to alternative conformations of the protein. It has also been shown that mutations in p53 found in a number of tumours correlates with destabilised tertiary structures (reviewed in Milner, 1995). The data regarding p53 mutations is conflicting in some areas. It has been observed that mutations in regions of p53 that should have no effect on proteasome mediated degradation result in enhanced stability. In the context of KIM-2 cells it is possible that p53 when bound to SV40 Tag is in a conformation which is more susceptible to calpain degradation than that mediated by the 26S proteasome. It has been observed that p53 cannot be degraded by the 26S proteasome when bound to DNA (Molinari

and Milner, 1995). In contrast to this the degradation of p53 by calpains was observed to be functional when p53 was either free or bound to DNA. One caveat of these studies is that p53 is a predominantly nuclear protein and calpains are cytoplasmic proteases. The mechanism whereby p53 is encountered by calpains is unclear but may relate to breakdown of the nuclear envelope during mitosis. This evidence would suggest that more than one pathway may contribute to p53 degradation and these may vary according to the cellular context and the physiological conditions encountered. It remains possible that the presence of SV40 Tag may favour p53 degradation by calpain. Examination of the effect that calpain inhibitors have on KIM-2 cells will resolve the question of whether this pathway can contribute to p53 degradation *in vivo*. Preliminary studies with a range of protease inhibitors, including those which inhibit calpains showed that only proteasome inhibition induced apoptosis in KIM-2 cells. If indeed calpain inhibitors can act to stabilise p53 in KIM-2 cells then this should induce apoptosis. Analysis of the cell cycle profiles and p53 expression levels in these cells will reveal whether they have elevated p53 protein.

In order to address the apoptotic response observed in more detail in these cells, careful dissection of each of the components of the G1/S phase of the cell cycle is required. Levels of G1/S phase expressed proteins should be examined following proteasome inhibition. This should address the question as to whether deregulation of one or a number of proteins drives this response. It will also generate information on the most likely candidates for inducing apoptosis. An important observation to come from these studies was the requirement for p53 in the apoptotic response occurring as a response to proteasome inhibition. To answer this question it is necessary to compare KIM-2 cells with another cell type e.g. the p53 wt and null ES cells. This will allow discrimination between p53 and other Tag binding proteins in this apoptotic response. It would be of interest to examine a cell line overexpressing p53 to determine if this can cause a similar type of apoptosis in these cells. p53 could be re-expressed in p53 null ES cells and the level of this protein examined following proteasome inhibition. This may go some of the way to explain

if the difference I observed in p53 levels following proteasome inhibition was dependent on the presence of SV40 Tag. More questions are required to address whether the presence of Tag alters the sensitivity of p53 to the 26S proteasome. mdm2 is the protein that normally acts to alter p53 stability and it would be of interest to examine whether the function of mdmd2 is altered by the presence of Tag. The apoptotic response induced also requires further characterisation. Of most interest would be the examination of p53 target genes e.g. p21, Bcl-2, Bax and caspase expression as all of these proteins have been suggested to be degraded by this complex. Examination of the downstream apoptotic pathway will reveal whether a conventional apoptotic response is initiated as a consequence of proteasome inhibition. Another major target of the 26S proteasome is E2F-1. We have shown that a component of this response is p53 dependent and I would like to examine the effects of rendering E2F-1 non-degradable. Altering the expression of this one protein is likely to alter the expression of a number of G1/S phase proteins e.g. cyclin A, cyclin E, p16, p107, pRb, c-myc, and E2F1. It is of interest to examine whether the response observed in cells overexpressing E2F-1 mirrors that seen following treatment with proteasome inhibitor and how the presence or absence of p53 effects this response. Inhibition of the 26S proteasome results in a high number of ubiquitinated proteins within the cell. Normally ubiquitination of proteins occurs as a stress response therefore it would be informative to examine stress activated pathways within the cell. Accumulation of these "stress signals" may trigger a response within the cell such as the activation of the stress activated kinase (SAPK) pathway. Prolonged activation of this pathway has been observed to induce apoptosis in some circumstances and therefore is one of a number of potential consequences of disruption of protein degradation.

The induction of a G1/S phase arrest followed by apoptosis in KIM-2 mammary epithelial cells in response to proteasome inhibition appears to be a complex response. The 26S proteasome has many substrates that are cell cycle regulated proteins. The timely expression of a number of these proteins acts to control the progression of the cell cycle. It follows that disruption of the expression

of these genes would alter the progression of the cell cycle. It is difficult to hypothesis that the deregulation of one particular substrate is the trigger for the apoptosis observed in these cells. It would appear much more probable that the apoptosis is occurring as a response to a number of conflicting signals to the cell e.g. during G1 levels of c-myc, E2F and p27 would all be high as a result of proteasome inhibition. These are signals which the cell was not programmed to encounter simultaneously. It is likely that when such a situation occurs the cell will be triggered to abort its cycle and die as a mechanism to prevent loss of cell cycle control, which could ultimately lead to neoplastic transformation.

Since the completion of the studies reported in this thesis a number of papers have been published concerning the apoptosis induced by proteasome inhibition. Bcl-2 is a member of a family of genes that act to regulate apoptosis. It is thought to suppress signals that induce apoptotic cell death. Bcl-2 has been shown to bind two proteins, one of which is Bax. Bax is 21% homologous to Bcl-2 and increased levels of expression of this protein appear to counteract the effects of Bcl-2 and promote apoptosis (Nunez and Clarke, 1994). Bcl-2 is thought to inhibit apoptosis activated by p53 by blocking a critical step that initiates the apoptotic cascade. Monney and colleagues (1998) have shown that overexpression of Bcl-2 in fibroblasts protected cells from apoptosis induced by decreased ubiquitination (due to an E1 mutation) in addition to treatment with proteasome inhibitor. They describe Bcl-2 as acting downstream of the accumulation of short-lived proteins as it did not prevent the overexpression of p53, p27, and cyclins D1 and B1. It is hypothesised that the accumulation of short-lived proteins may act to inhibit survival factors that are crucial to the cell. Conversely Bcl-2 may compensate for the absence of other survival factors which are substrates of this pathway e.g. NFκB. Bcl-2 has been shown to prevent apoptosis in response to a wide range of signals (Sentman *et al.*, 1994 and Cuende *et al.*, 1993). Mdm2 and Bax have also been observed to be substrates of the 26S proteasome (Chang *et al.*, 1998). Both of these genes are regulated by p53; p53 induction of Bax induces apoptosis and mdm2 alters the sensitivity of p53 to degradation by the proteasome. The increase in the stability of

these proteins following proteasome inhibition was observed to occur independently of p53. This would suggest that the proteasome normally acts to negatively regulate apoptosis by degrading the downstream target of p53, Bax. It was observed that Bcl-2 levels are not altered following proteasome inhibition. Phosphorylation of Bcl-2 inactivates the ability of Bcl-2 to prevent apoptosis suggesting that a minimum of two mechanisms act to regulate the control of apoptosis by Bax, ubiquitination of Bax and phosphorylation of Bcl-2. It would be of interest to examine the levels of Bcl-2 and Bax in KIM-2 cells to determine if they are stabilised in response to proteasome inhibition. Clearly a different mechanism of death is induced in these cells as p53 was a definitive requirement for an apoptotic cascade. If Bcl-2 and Bax levels were the only factors controlling apoptosis in KIM-2 cells then death should have been observed at 33°C in addition to 37°C. Chang and colleagues (1998) described an independence of p53 in this response that conflicts with the observations detailed in this thesis.

The 26S proteasome degradation pathway has been linked to the function of heat shock proteins (Hsp), which increase cell tolerance to stresses. Hsp72 has been observed to bind damaged or misfolded proteins and acts either to facilitate the repair of the protein or to target it to the 26S proteasome. Another pathway that is activated in a similar manner is the c-Jun terminal kinase (JNK) signalling cascade. Prolonged activation of JNK is harmful to the cell and ultimately leads to the induction of apoptosis. Hsp 72 interferes with the activation of JNK and functions to prevent apoptotic signalling under certain stresses (Gabia *et al.*, 1997). Meriin and coworkers (1998) have recently described activation of the JNK signalling pathway and induction of Hsp72 in response to MG132 treatment *in vitro* followed by the induction of apoptosis. This effect could be abrogated when the JNK signalling cascade was suppressed suggesting that the proteasome mediates its apoptotic response by a JNK pathway. Given that so much conflicting information is available with regard to apoptosis induced by proteasome inhibition more research is required to clarify the precise pathway which is activated in response to this insult.

7.4 26S Proteasome Implications in Disease

The ubiquitin-proteasome degradation pathway has a vast number of substrates that are involved in many fundamental biochemical processes. This results in this pathway becoming a prime target for involvement in cancers or diseases arising from deregulation. The 26S proteasome pathway has recently been implicated in a number of neurodegenerative diseases. Transgenic mouse models of Huntingtons Disease (HD) have been observed to develop neuronal intranuclear inclusions that contain ubiquitin proteins (Davies *et al.*, 1997). Increases in both the proteasome and ubiquitin had also been observed in dystrophic neurites in Alzheimer Disease (AD) and Parkinson's Disease (Ii *et al.*, 1997). The amyloid β protein, which has been implicated in AD, has been shown to bind to the inner catalytic sites on the 20S proteasome (Gregori *et al.*, 1997). This may account for the increase in ubiquitinated proteins observed in many neurodegenerative diseases but it is unclear at present whether a reduction of proteolysis occurs as a result of the disease or whether it contributes to the disease.

The HIV-1 Tat protein has been observed to interfere with the 26S proteasome pathway. The Tat protein causes a number of different effects during acquired immunodeficiency syndrome (AIDS) which determine infectivity and cytopathicity in the developing infection. Seeger and colleagues (1997) have observed Tat binding to both the 19S regulatory complex and the 20S proteasome. Tat was shown not to bind to the 11S regulator and was shown to compete with the 11S subunit for binding to 20S proteasomes. Binding to the 20S proteasome by Tat inhibits this 20S/11S complex while stimulating 26S proteasome activity. *In vitro* the effects of Tat on the proteasome system may result in a decrease in antigen processing and reduced MHC class I presentation in infected cells. Tat also displays an immunosuppressive activity on lymphocytes, which in concert with its effects on

the proteasome pathway may contribute to the profound immunodeficiency seen in AIDS patients.

The proteasome has additionally been described to function in events mediated by oncogenic viruses (Scheffner *et al.*, 1990). In HPV-related cancers the oncogenicity of the papilloma virus is mediated by upregulation of p53 degradation by the proteasome. The E6 protein of high risk HPV (e.g. HPV-16, -18, -5 and -8) binds to p53 and mediates its ubiquitin dependent degradation and this step is crucial for immortalisation of human cells by HPV. Low risk HPV (e.g. HPV-6 and -11) encodes an E6 protein that cannot bind to p53 and therefore does not promote its degradation. Alterations of p53 stability by the 26S proteasome may be functionally equivalent to p53 inactivation, which is observed in a high number of cancers.

Progression through the cell cycle is promoted by the activity of the proteasome in addition to cdks. The cdk inhibitor p27, which is a substrate of the 26S proteasome is emerging as a potential clinical marker. Low p27 protein levels have been found in a number of common tumours e.g. colorectal carcinomas and breast cancers. This is generally associated with a poor prognosis. In the case of breast cancer Catzavelos and co-workers (1997) have shown that increased proteolysis of p27 can be an early event in tumourigenesis. Cyclins B, D1 and E have all been shown to be overexpressed in breast cancers but it is unclear whether this is due to a decrease in proteolysis (reviewed in Spataro *et al.*, 1998). These initial studies suggest that examination of the 26S proteasome pathway in situations where deregulation of cell cycle related proteins are observed may show that proteasome dysfunction contributes to the progression of these diseases.

The proteasome is also implicated in events that control gene transcription. The proto-oncogene products c-Jun and c-Fos constitute the transcription factor AP-1 either as heterodimers or c-Jun homodimers. Both of these proteins are substrates of the ubiquitin-proteasome pathway. The degradation of c-Jun is determined by a region called the delta domain that is absent in its transforming retroviral counter-

part v-Jun. The increased stability of v-Jun is thought to contribute to its oncogenicity (Treier *et al.*, 1994). The transcription factor AP-1 has recently been implicated in multidrug resistance, which also has a link to the proteasome. The yeast homologue of AP-1, Pap-1 confers multidrug resistance through the Pad-1 protein. The human homologue of Pad-1, termed POH-1, has been shown to have homology to subunit 12 of the regulatory complex (Dubiel *et al.*, 1995b). It has subsequently been shown that this protein is present in the 26S proteasome. POH-1 also displays sequence similarity to JAB-1, which interacts with c-JUN to activate AP-1. Spataro and colleagues (1998) propose that proteasome subunit POH-1 could act to upregulate AP-1 factors and confer multidrug resistance on the cell. As the proteasome pathway is highly conserved in mammals, it may act to confer drug resistance to anti-cancer agents *in vitro* and could potentially be involved in drug resistance in human tumours.

DNA repair pathways are regulated by ubiquitin-proteasome activity. In *S. cerevisiae* expressing a mutant Rad6 DNA repair enzyme, defects in ubiquitin conjugation was observed suggesting this enzyme has an E2 function. Further evidence supporting this came from studies on Rad23, which have shown that the ubiquitin-like region in this protein was essential for its function (Watkins *et al.*, 1993). Yeast mutants in E1 enzymes also display defects in DNA repair in response to UV induced DNA damage suggesting that the proteasome pathway is strongly implicated in DNA repair. At present it is unclear whether the entire pathway is involved or whether the ubiquitin binding proteins directly interact with the DNA repair pathway. The deubiquitinating enzymes may also play a potential role in oncogenic transformation. The human Ubp Tre-2 has been found to be tumourigenic when expressed at high levels (Papa and Hochstrasser, 1993)

The 26S proteasome mediated degradation pathway has implications in a number of diseases and for these reasons it may represent a target for therapeutic intervention. To date, the best-known proteasome inhibitor is lactacystin, a *Streptomyces* metabolite. Lactacystin has been observed to inhibit cell cycle

progression in human osteosarcoma cells (Fenteany *et al.*, 1994) and to induce apoptosis in a number of other cell types. It is not clear whether this drug has any anti-tumour effects. The majority of the other proteasome inhibitors available currently are peptide aldehydes, which can also induce apoptosis in human tumour cell lines (Fujita *et al.*, 1996). The proteasome displays a very diverse range of substrates and any attempt to target these non-specifically might be associated with toxicity. The rapidly expanding knowledge of this proteolytic pathway in both normal and disease processes may soon provide the information necessary to generate a proteasome-targeting drug. The complexity and specificity of the proteasome may dictate that specific inhibitors of individual proteasome-mediated processes may be required for any pharmacological intervention to the therapeutically advantageous.

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